European Community Reference laboratory
for monitoring bacteriological and
contamination of bivalve molluscs

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Community Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Molluscs, Cefas, Weymouth

Technical Report for Calendar Year 2008

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Technical Report for calendar year 2008

Legal functions and duties

The functions and duties of the CRL are specified in Article 32 of Council Regulation (EC) 882/2004 (Official Journal of the European Communities No L165).

Introduction

The annual work programme for the CRL for 2008 was approved by the European Commission in November 2007. This report details consequential activities of the CRL according to the work programme 2008 (Annex I), additional tasks described under the resolutions of the 7th workshop of microbiological NRLs held in Weymouth 2008 (Annex II) and other responsibilities outlined in Council Regulation (EC) 882/2004 for the calendar year 2008.

1. Scientific advice and support

To the European Commission.

The CRL provided advice to DG SANCO in the form of reports, briefing documents, presentations and discussions on *inter alia* the scientific basis of the application of sanitary surveys under Regulation (EC) No. 854/2004 to new and existing harvesting areas; approaches to classification and monitoring of marine gastropods, tunicates and echinoderms; methodologies for the detection of histamine; the Advisory Committee on Fisheries and Aquaculture (ACFA) WG2 " aquaculture"; the FAO/WHO report "viruses in foods"; pathogenic *Vibrio* spp. associated with bivalve molluscan shellfish (BMS) and the potential impact of the repeal of the Shellfish Waters Directive (79/923/EEC) and its incorporation into the Water Framework Directive (2006/60/EC). The CRL also gave specialist assistance to the FVO division specifically in the provision of national experts on missions to Thailand and as invited speakers on FVO training courses. In addition the CRL met with colleagues at the Joint Research Council at Institute for Reference Materials and Measurements (IRMM) in Geel, Belgium to discuss a potential collaborative project to develop reference materials in support of the forthcoming norovirus and hepatitis A reference standard.

The CRL has also given considerable assistance to NRLs, third countries and members of EFTA on calculation of MPNs and interpretation of MPN tables, on marine gastropods, echinoderms and tunicates, monitoring plans, application of sanitary surveys and on technical requirements

for depuration and conditioning plants. The CRL continued to work very closely with NRL France on the validation of the impedance method using Bactrac 4300 for enumeration of *Escherichia coli* in BMS according to ISO 16140 through a series of meetings, presentation and review of the final reports. In December the CRL recommended that the validation programme was carried out in accordance with the requirements of EN ISO 16140 (Microbiology of food and animal feeding stuffs - protocol for the validation of alternative methods), as required by EC 2074/2005 and EC 2073/2005, that the results were satisfactory and therefore that the validation had been completed successfully. The CRL was a member of the expert working group convened by MicroVal considering the validation of a pour plate based method for the enumeration of *E. coli* (ISO 16649-2) in BMS in the Netherlands. A full register of advice provided by the CRL is available from the CRL co-ordinator on request.

Other Scientific activities

In 2008 the CRL active in a number of initiatives at ISO SC9 and CEN WG6 "Microbiology of food and animal feeding stuffs" The CRL continued to chair the CEN/TC 275/WG6/TAG4 expert working group on viruses in foods developing a horizontal method for the detection of norovirus and hepatitis A in bivalve molluscs. In 2008 the method for viruses (norovirus and hepatitis A) in foodstuffs, including bivalve molluscs has been finalised through targeted experimental work between members of TAG4. One meeting was held over 2 days in London where the final text of the document was agreed. It was agreed in principle that both a quantitative and qualitative standard would have utility, and as a result a part 2 document describing qualitative determination has also been drafted for consideration by the group. The quantitative part of this draft standard will be presented at CEN WG6 in the Spring of 2009. As designated project leader under CEN "Methods on analysis of foodstuffs concerning food hygiene" (M/381) in support of EU Food Hygiene Regulations the CRL awaits progress on the formal validation of this developed methodology. The method is now in routine use at the CRL. The CRL continues to play an active role in research and development in the area of marine *Vibrio* spp. associated with bivalve shellfish. As project leader for *Vibrio* spp. molecular methodology development the CRL presented framework documents outlining a two part standard comprising nucleic acid hybridisation and real-time PCR approaches to the enumeration and rapid detection of pathogenic strains of *V. parahaemolyticus* directly from seafoods. The CRL is also the designated project leader for the validation of two cultural methods for the detection of human pathogenic vibrios in seafoods including BMS under M/381 (ISO TS 21872 parts 1 and 2) and as with viruses awaits progress on the mandate. The CRL was an active participant in SC9/WG3 "Microbiological validation" reviewing ISO 16140 for the validation of alternative methods attending one meeting and co-authoring part 1 of the revision "Terminology". In addition the CRL

has contributed methods suitable for the analyses of shellfish to the new technical advisory group considering sampling (CEN/TC 275/WG6/TAG6). The CRL has undertaken further meetings and discussions with the European Mollusc Producers Association (EMPA) with regard to their proposal for a pollution alert system (SUMO). SUMO representative presented the proposed project to the CRL in February 2008 and were given the opportunity to discuss the programme with members of the NRL network following the 7th annual workshop in May. The CRL has acted as an independent scientific and technical reviewer for the proposed work programme. CRL staff also participated in a US EPA workshop equivalency of classical and molecular based methods in water quality.

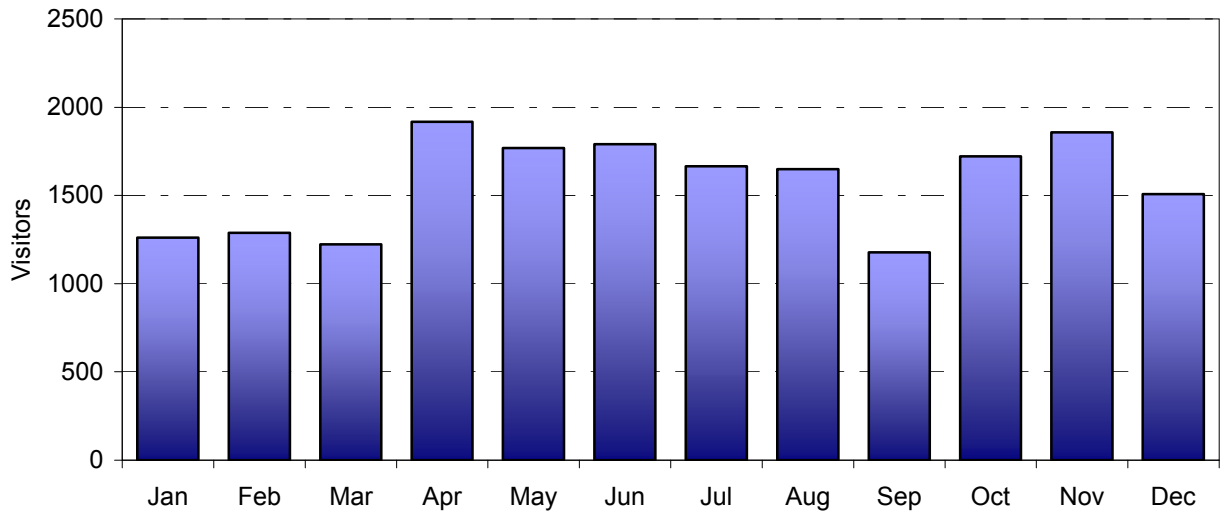
CRL staff presented research at a number of high profile international scientific conferences including the International Coastal Water Symposium, UK, 5th SEAFOODplus, The Netherlands, Cost 929 symposium "Current developments in food and environmental virology", Italy ASSG, Scotland and Society for General Microbiology, UK.

2. Co-ordination of activities of NRL network and provision of technical assistance and training (including third countries)

Website

The CRL website www.crlcefas.org provides an effective means of dissemination of information and continues to be a very valuable resource. During 2008 the website was regularly updated to reflect CRL peer-review publications, reissued CRL standard operating procedures, CRL proficiency testing reports. Notably a new section comprising all presentations, working groups and recommendations following the first International Workshop on the Application of Sanitary Surveys held in Weymouth in September 2008. All reports and publications are public domain information, documents restricted to NRLs and other stakeholders require prior registration and log on for access. User statistics for the CRL website for 2008 are given in Figure 1. In 2008 users registered an average 1736 visits per month representing a 35% rise in visitor usage compared with 2007.

Average activity by month in 2008 (visitors)



Note. Figure 1 shows the number of individual visitor sessions recorded on the website per month between January and December 2008. A session reports individual users for any given time interval. Sessions are tracked per IP address and must register at least one hit to be included. A hit is defined as any request for data such as a web page, bitmap, CGI gateway or file.

NRLs workshop May 6-8 Weymouth, UK.

The CRL hosted the seventh workshop of European NRLs on 6-8 May. Thirty-five experts representing NRLs from 20 Member States, Croatia, Iceland and Norway attended. In addition four observers from the Ministry of Maritime Affairs and Fisheries, South Korea were present. Discussion focused on supervision of official control laboratories, microbiological methods for statutory determinands, sanitary surveys and monitoring, analytical tolerance, depuration, proficiency testing, viruses and vibrios. A full report of the 7th workshop including copies of all of the presentations and associated meeting papers are available on the CRL website (www.crlcefas.org). Thirty formal resolutions were passed at the workshop. The workshop resolutions are included as Annex II of this report and the workshop agenda as Annex III.

Training and technical assistance

Formal workshops

Together with the US Food and Drug Administration (FDA), the CRL co-hosted the 1st International workshop on the application of sanitary surveys. The workshop programme is included as Annex IV. The aim of this unique international event was to enhance the

understanding of both technical aspects and the equivalence of the various approaches to the application of sanitary surveys, operated by countries with significant bivalve shellfish production across the globe. For example, those contained within the Model Ordinance of the U.S. FDA National Shellfish Sanitation Program, Guide for the Control of Molluscan Shellfish, 2005 and the requirements outlined in EU Commission Regulation (EC) No 854/2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption, further described in the EU CRL publication: Microbiological monitoring of Bivalve Harvesting Areas, Guide to Good Practice: Technical Application.

The workshop will brought together experienced specialists from both a technical scientific and regulatory background from EU, US, New Zealand, Australia, Chile, Mexico, Korea and China. The workshop comprised both oral presentation and fieldwork, including practical demonstration activities. It was considered successful in achieving its major objectives of sharing expertise, approaches to problem solving and establishing a network of excellence for future collaboration in this exciting area. A follow-up work to build on these achievements is planned for 2010. The workshop delegates agreed a number of recommendations these are included as Annex V of this report.

Provision of technical assistance and reference materials

Additional technical assistance was supplied in the form of provision of CRL standard operating procedures and laboratory protocols. Reference materials were distributed to NRLs in Belgium, The Netherlands, Norway, and Portugal and laboratories in Croatia and South Korea. Shellfish adapted Enterobacteriaceae were supplied to the Health Protection Agencies Food EQA unit as part of an ongoing collaboration on provision of proficiency testing programmes

3. Proficiency testing and quality assurance

Participation of MS NRLs, accession, and third countries in CRL organised proficiency testing (PT) in 2008 is summarised below and tabulated in Annex VI. PT (ring trial) reports are presented in Annexes VII, VIII, IX, X and XI. PT reports are presented at the annual workshop of NRLs and are also available on the CRL website www.crlcefas.org

E.coli / *Salmonella* External Quality Assurance Scheme (EQA)

The CRL collaborates with the UK Health Protection Agency (HPA) on the application of a Shellfish External Quality Assurance (EQA) Scheme for proficiency testing among laboratories analysing BMS. The Shellfish EQA scheme is targeted at analysis of the statutory determinants *E. coli* and *Salmonella* spp. in BMS and is the primary means of ensuring comparative performance by NRLs. Numerical performance assessments for 3 EQA distributions between

March 2008 and November 2008 are included in this report and summarized in Table 1. A full report of laboratory performance is available from the CRL co-coordinator.

Table 1. Performance assessment for *E. coli* MPNs for samples SF0066 - SF0071

	Distribution								
	SF029		SF030		SF031		Cumulative score	Max possible score	%
Lab no.	SF0066	SF0067	SF0068	SF0069	SF0070	SF0071			
121	12	12	12	12	12	12	72	72	100
391[a]	12	12	NE	NE	12	12	48	48	100
403	12	12	12	12	12	12	12	72	100
493[a]	NE	NE	NE	NE	12	12	24	24	100
583	12	12	12	12	12	12	72	72	100
593	12	10	12	12	-	-	46	72	64
594	12	12	12	12	12	12	72	72	100
596	12	12	10	12	12	12	70	72	97
597	12	12	12	12	12	12	72	72	100
601	12	9	12	12	12	9	66	72	91.7
604	12	6	12	12	12	12	66	72	91.7
605	0	0	0	0	12	9	21	72	29
629[a]	12	12	NE	NE	NE	NE	24	24	100
649	7	7	12	12	12	12	62	72	86
651	12	12	12	12	12	12	72	72	100
653	12	12	12	12	12	12	72	72	100
658	12	12	12	12	12	7	67	72	93
660[a]	12	12	0	0	NE	NE	24	48	50
662[a]	12	12	12	12	NE	NE	72	72	100
701	7	4	12	12	12	12	59	72	81.9
703	12	12	12	12	12	12	72	72	100
704[a]	12	9	NE	NE	NE	NE	21	24	87.5
715[a]	12	12	NE	NE	NE	NE	24	24	100
718	12	12	10	12	2	2	50	72	69
719	12	12	12	12	12	9	69	72	95.8
720	12	12	12	12	12	12	72	72	100

[a] Laboratories not subject to full performance assessment

Twenty-six NRLs or laboratories of equivalent status registered were registered for the CRL/HPA EQA Shellfish as of January 1 2008. Designated NRLs Latvia, Hungry and Bulgaria did not participate in the scheme. Laboratories 629, 660, 704 and 715 formally left the scheme in 2008. Nineteen NRLs returned results for three distributions and were thus subject to a full performance assessment. Of these 2 NRLs achieved cumulative scores over three distributions <70%. Laboratories were contacted and referred to troubleshooting information.

E. coli and *Salmonella* spp. whole animal ring trial

In November 2008 the CRL organised a distribution PT using matrix samples to examine initial sample preparation and preparation of dilutions) i.e. aspects not challenged by using laboratory constructed samples. Fifty-four laboratories (20 NRL and 34 non-NRL Official Control laboratories) participated in the scheme, non NRLs were charged on a cost recovery basis for the distribution.

Participants examined a single sample of Common mussels (*Mytilus edulis*) for the statutory determinands (*E. coli* and *Salmonella* spp). Reported *E. coli* MPN values were compared to the median of all participants' results. Upper and lower acceptability limits were calculated as the participants' median ±3 standard deviations (SD) and ±5 SD (≡95% and 99% confidence limits respectively) SD calculations were based on the expected inherent variability of the five-tube MPN method. Performance assessment was performed according to the CRL/HPA EQA Shellfish scheme for a single distribution, with modified to reflect replicate analyses of a single sample.

Ninety-six percent of laboratories returned duplicate *E. coli* MPN results within the anticipated range for *E. coli* (96%). Fifty-three laboratories from the fifty-four returning results reported the use of the *E. coli* reference method (ISO TS 16649-3) or Donovan *et al* 1998. One laboratory reported the use of NMKL 96 for enumeration of *E. coli.* It is noted that this method has not been formally validated according to ISO 16140 for bivalve shellfish.

Only one laboratory detected *Salmonella* spp in a single replicate. *Salmonella* spp. was detected in a third of the reference samples however it is considered likely that this was associated with disproportionate exposure of reference samples to faecal contamination in the harvesting area. Further details can be found in the full PT report (Annex VII). Seventy-six percent of laboratories used the EU specified reference method for *Salmonella* spp (ISO 6579).

Norovirus and Hepatitis A ring trial

The CRL distributed stabilised NoV (GI and GII derived from faecal material) and HAV (tissue culture HM175 strain) to 29 participating laboratories (18 MS NRLs, 3 in-country testing laboratories, 1 EFTA NRL, and 8 third country laboratories including laboratories in the U.S., New Zealand, Canada, South Korea, Hong Kong and Chile). Samples contained moderate to low titre of virus to mimic environmental levels. A full report performance is included as Annex VIII (RT25). In brief 27 (93%) laboratories returned results to the CRL. Approximately a third of laboratories returned correct results for all samples. False positive reporting rates were low (1-

4%), false negative rates were higher ranging from 15 to 29%. Participants' correctly assigned samples as HAV positive than either norovirus genotype. Seventeen laboratories returned quantitative results compared with fifteen in 2007.

Vibrio parahaemolyticus ring trial

The CRL distributed 6 PT samples for detection of total and potentially pathogenic V. parahaemolyticus in February 2008. Twenty-two laboratories registered for the scheme, 16 NRLs and 6 others including laboratories in Canada, Croatia, Iceland, Norway, Turkey and the US. Overall laboratory performance was good well. A false negative rate of 6.8% was observed for identification of *V. parahaemolyticus*. The false positive rate was less than 1%. Eighteen laboratories used methods that enabled detection of pathogenicity markers. The majority of laboratories applying these tests correctly assigned the presence or absence of both *tdh* and *trh*. The full report of laboratory performance is included as Annex IX.

4. Confirmatory testing

During 2008 the CRL maintained accreditation to ISO 17025 for the following methods:

- Examination of shellfish for *Salmonella* spp.
- Enumeration of *Escherichia coli* in bivalve molluscan shellfish
- Detection of *Vibrio parahaemolyticus* in bivalve molluscan shellfish
- FRNA bacteriophage enumeration bivalve molluscan shellfish.

5. Development of analytical methods

The CRL continued to undertake extensive practical research and development in support of initiatives at CEN. All outstanding technical issues relating to the proposed standard for quantitative determination of norovirus and hepatitis A were resolved and the method completed. In parallel limited practical work continued to generate data to assist in the development of standardised methods for pathogenic vibrios. Further homogeneity and stability data was generated on lenticules as viral carrier as reference and proficiency testing materials.

Rachel Rangdale (March 2009)

LEGAL FUNCTIONS AND DUTIES

The functions and duties of the CRL are specified in Article 32 of Regulation (EC) No 882/2004 (Official Journal of the European Communities No L 165 of 30.4.2004).

In the 2008 work programme year 27 Member States and 3 candidate countries (Croatia, Turkey and Former Yugoslav Republic of Macedonia) are considered eligible for CRL assistance and invited to participate in CRL organised training programmes, ring trials/external quality assessments schemes etc. The full integration into the European Union of recent accession Member States continues to be a priority area, and is facilitated via the provision of additional advice, training and assistance.

WORK PROGRAMME, 2008

	Duration
1.Scientific advice and support	
1.1 Assist DG Sanco in functioning and implementation of Community food hygiene legislation, e.g. drafting guidance documents, consideration of analytical tolerances, etc.	10 days
1.2 Participate in relevant EU and International scientific committees (ISO/CEN, WHO/FAO, ICMSS etc). In 2008 the CRL will:	45 days
• Chair and co-ordinate the activities of the CEN/TC 275/WG6/TAG4 developing a CEN standard for detection of norovirus and hepatitis A in foodstuffs, including bivalve molluscs.	
• Lead and co-ordinate the activities of CEN/TC 275/WG6/TAG3 in the elaboration of molecular based enumeration methods for pathogenic marine vibrios in bivalve shellfish (see resolution 33, 6th workshop of NRLs).	
• Participate in ISO/TC34/SC9/WG3 working group on validation of methods (revision of EN ISO 16140) to include the elaboration of ISO technical report on recommendations for establishing/revising reference methods.	
1.3 Assist DG Sanco with specialist assistance in relation to food and veterinary inspections of Member States, Accession Countries and Third Countries and with other trade issues (e.g. equivalency negotiations, industry initiatives such as SUMO) as they arise.	15 days
1.4 Co-operate with, and assist DG TAIEX in the provision of training and advice to Accession Counties.	4 days
1.5 Undertake CRL missions in support of the above activities.	35 days
• During 2008 missions are foreseen in relation to the annual	

	meetings of ISO and CEN (up to 2 missions); the CEN/TAG4 working group on viruses in food (2 missions); CEN/TAG3 working group on vibrios (2 missions); ISO/WG3 working group on validation of methods (2 missions) and up to 3 missions in support of NRLs and DG Sanco activities.	
1.6	Participation in relevant international scientific conferences	5 days
1.7	Participate as a member of the steering committee in the ICMSS' international forum on harmonisation of approaches to bivalve shellfish sanitation, including standardisation of methodologies for indicator organisms, and human pathogenic viruses and bacteria.	3 days
1.8	Participation in the joint FAO/WHO/Codex Task Force undertaking risk profiling and formulating Food Risk Management Guidance on noroviruses in food, including bivalve shellfish.	5 days

2. Co-ordination of activities of NRL network and provision of technical assistance and training

2.1	Participate in annual CRL Directors co-ordination meeting and other CRL co-ordination meetings/workshops as appropriate	5 days
2.2	Organise, host, and participate in the seventh annual NRL workshop, produce resolutions and other workshop outputs (May 2008, CRL Weymouth). To include CRL administrative assistance.	45 days
2.3	Undertake CRL activities and commitments agreed in resolutions at annual workshops (as posted on www.crlcefas.org).	Up to 100 days
2.4	Organise specialist, targeted, practical training for NRLs, MS competent authorities and the FVO on sanitary surveys - in accordance with the requirements of 854/2004 on official controls.	8 days
2.5	In collaboration with the US FDA organise and host a joint workshop on implementation and approaches to sanitary surveys in the EU and US.	15 days
2.6	Supply specialist information and advice on bacteriological and viral methods to NRLs (particularly new MS NRLs and accession countries), Official Control testing laboratories, and third county laboratories. To include assistance on implementation of methods, accreditation to IEC ISO17025, validation of alternative methods according to ISO16140, provision of CRL SOPs and transfer of other technical information.	10 days
2.7	Provide specialist training and/or training courses to NRLs, accession country NRLs and others in relation to analyses of *E.coli*, *Salmonella* spp., *Vibrio* spp., FRNA bacteriophage, Norovirus, hepatitis A virus and other aspects of bivalve shellfish hygiene as required.	5 days

2.8	Continue to update and improve the CRL website (www.crlcefas.org) as a primary means of dissemination of information to NRLs and others.	7 days

3 Ring trials, comparative testing and quality assurance

3.1	Organise comparative (proficiency) testing for NRLs for *E.coli* and *Salmonella* spp. in bivalve molluscs via the CRL/HPA shellfish EQA scheme (see resolution 5 of 6th workshop of NRLs). Analyse results, produce report, advice and recommendations (by May 08).	40 days
3.2	Organise Norovirus and hepatitis A ring trials (see resolution 26 of 6th workshop). Analyse results, produce report and recommendations (by May 08).	50 days
3.3	Undertake *Vibrio parahaemolyticus* ring trials appropriate for methods enabling enumeration of pathogenicity principles (thermostable direct and thermostable direct related haemolysins) (see resolution 32 of 6th workshop). Analyse results, produce report and recommendations (by May 08).	50 days
3.4	Undertake FRNA bacteriophage proficiency testing. Extend provision of EQA material, methods of analysis etc to national testing laboratories if required by NRLs (see resolution 4 of 6th workshop).	15 days
3.5	To challenge aspects of the *E. coli* and *Salmonella* spp. methods not covered by the standard shellfish EQA scheme organise a whole animal ring trial (see resolution 7 of 6th workshop) for NRLs, the scheme will be extended to selected Official Control Laboratories. Analyse results, produce report, advice and recommendations (by May 08).	30 days
3.6	Prepare stable reference material using biological LENTICULE carriers for norovirus and Hepatitis A (see resolution 28 of 6th workshop). Perform homogeneity and stability analyses. Distribute data and LENTICULES to NRLs for use as control material on request.	30 days

4 Confirmatory testing

4.1	Maintenance of CRL laboratory competence and expertise on analytical methods for monitoring virological contaminants of bivalve molluscs (Norovirus and hepatitis A virus). To include maintenance of IEC ISO 17025 accreditation for detection of norovirus in bivalve shellfish and adoption of CEN method for viruses.	70 days
4.2	Maintenance of CRL laboratory competence and expertise on analytical methods for monitoring bacteriological contaminants of bivalve molluscs (*E.coli*, *Salmonella* spp., FRNA bacteriophage, marine vibrios). To include maintenance of IEC ISO 17025	70 days

	accreditation of enumeration of *E. coli* and FRNA bacteriophage and the detection of *Salmonella* spp. and *Vibrio parahaemolyticus*.	
4.3	Contribution to costs of the maintenance of CRL capability to perform analysis for marine vibrios in bivalve molluscs other than *V. parahaemolyticus.*	10 days
4.4	Performance of above tests on outbreak material or on occasion of disputed test results (on request of DG Sanco).	Included in above

5 Development of analytical methods (undertaken at CRL)

5.1	Contribution as the project leader towards the validation of the TAG4 reference method for the detection of viruses in food (CEN/TC 275/WG6/TAG4). Note. Validation of the TAG4 reference method for viruses in foods, including bivalve shellfish is a priority area. In the event of CEN mandate M/381 "Methods of Analysis of foodstuffs concerning food hygiene" (Mandate for standardisation M/381 addressed to CEN) not being fully funded, resources will be diverted from other areas within the work programme to enable validation of the virus method for bivalve shellfish.	15 days
5.2	Contribution as the project leader towards the elaboration and validation of the TAG3 molecular based standard for the detection of potentially pathogenic vibrios in foodstuff, including bivalve shellfish using molecular methods - both nucleic acid hybridisation and real time PCR approaches.	20 days
5.3	The existing *E.coli* enumeration reference method ISO TS 16649-3 specified in Commission Regulation (EC) No 2073/2005 is published as a technical specification with an expiry of December 2008. It is proposed that the CRL, in collaboration with the NRL network undertakes work to generate data enabling its adoption as a full horizontal standard. Thus enabling it to remain as an ISO recognised method for *E. coli* enumeration in live bivalve molluscs, tunicates, echinoderms and marine gastropods in Regulation.	50 days

Rachel Rangdale
CRL Co-ordinator
August 2007

Annex 1

Resources necessary to fulfil the listed activities

I. LEGAL FUNCTIONS AND DUTIES

The functions and duties are specified in Articles 3 and 4 of Council Decision 1999/313/EC (Official Journal of the European Communities No L 120 of 8.5.1999).

PROGRAMME FOR THE PERIOD JANUARY – DECEMBER 2008

Item	Baseline Activity	Resources required (Euros)	7% overheads	TOTAL budget requested
1	Scientific advice and support	31,515.20	2,206.06	33,721.26
2	Co-ordination of activities of NRL network and provision of technical assistance and training	47,272.80	3,309.10	50,581.90
3	Ring trials, comparative testing and quality assurance	157,576.00	11,030.32	168,606.32
4	Confirmatory testing	78,788.00	5,515.16	84,303.16
5	Development of analytical methods (undertaken at CRL)			
	Total Baseline Costs	**315,152.00**	**22,060.64**	**337,212.64**
	Workshop	45,000.00		45,000.00

Resolutions of the 7th workshop of Microbiological NRLs for Bivalve Molluscs, Weymouth, UK, 6-8th May, 2008

Supervision of official control laboratories

1. NRLs provided information to update the "Questionnaire to laboratories, 2007". The CRL agreed to update information on bivalve mollusc production, methodology, accreditation and supervision of Official Control Laboratories and post a summary on the CRL website.

Microbiological methods - statutory determinands

2. Further to resolution 25 of the 6th workshop in 2007, and in the absence of additional data from other matrices, it was agreed that the CRL should write to ISO SC9 requesting an extension for ISO TS 16649-3 (*E. coli* reference method) to ensure its continuation as a technical specification. NRLs were encouraged to support the generation of data for matrices other than bivalve molluscs to facilitate its adoption as a full ISO standard method.

3. When deriving *E. coli* MPN values for Official Control testing it was agreed to only accept MPN combinations falling into probability categories 1 (95%) and 2 (4%) as this is an additional quality control check. The CRL provided MPN tables with these probability categories annotated and agreed to work towards updating the 5x3 MPN tables in ISO 7218:2007.

4. The NRLs recognised the comprehensive work undertaken by NRL France on validation of the impedance method (Bactrac 4300) according to ISO 16140. It was noted that following submission of a final report the CRL intended to issue a formal response copied to NRLs and the Commission.

5. NRLs supported the proposal that, if the Bactrac 4300 methodology was found to be equivalent to the *E. coli* reference method (ISO TS 16649-3), a recommendation would be made to DG Sanco to consider formal recognition of the use of the methodology for Official Control testing through a Commission Decision.

6. NRL Netherlands reported on the continued use of ISO16649-2 (TBX method) for Official Control testing in the Netherlands on the instruction of the competent authority. The NRL Netherlands advised that they do not consider the TBX method to be validated according to the requirements in EU Food hygiene legislation and therefore should not be used.

Microbiological monitoring - sanitary surveys and monitoring

7. The workshop noted that there was a lack of systematic activity with respect to the application of sanitary surveys across the Eustis was partially because Member State sanitary survey programmes were based on the previous Commission advice that the sanitary survey provisions of Regulation 854/2004 only applied to newly classified areas. It was identified that this was not clearly stated in the Regulations and the Commission undertook to seek a legal opinion which would be circulated to NRLs when available.

8. The workshop supported the proposal for more formal adoption of the CRL guidance for microbiological monitoring of bivalve mollusc harvesting areas (published on the CRL website). The Commission considered that the format of the 'principles' document would need revision for coherence with other Commission guidance and would advise the CRL on the next steps to progress the document.

9. The CRL informed the workshop that the response rate by NRLs on the questionnaire on monitoring for echinoderms, gastropods and tunicates had been poor. The Commission advised this was important information. The CRL therefore agreed to recirculate the questionnaire among

non-responding NRLs who agreed to give this a higher priority. The CRL agreed to produce a dossier of information and lodge it on the website.

Microbiological monitoring - analytical tolerance

10. With reference to the standards for classification of production areas in EU Regulation 854/2004, NRLs reaffirmed their opinion that scientific justification for application of tolerance based upon analytical uncertainty for category B sites applied equally to class A (and C areas).

11. Further to the above, NRLs noted that the lack of information and guidance in the legislation on how to deal with non-compliant class A results caused significant problems for competent authorities in the classification of harvesting areas. The CRL agreed to seek clarification from the Commission and report back to NRLs.

12. The Commission advised NRLs that their working group on implementing measures had considered the 10% tolerance for class B areas (as included in transitional arrangements 1666/2006 - expiry 2009) and currently considered that it should be retained subject to a maximum result upper limit of 46,000 MPN *E. coli*/100g.

Depuration

13. Further to resolutions 17 and 21, of the 5th and 6th workshop in 2006 and 2007, NRLs expressed support for the elaboration of an EU guidance document covering commercial depuration practices with respect to removal of bacteriological contamination.

Proficiency testing for statutory determinands

14. NRLs agreed to maintain commitment to the CRL/HPA EQA as the primary means of proficiency testing for *E.coli* and *Salmonella* spp. amongst the NRL network.

15. The network noted the improvements over recent years, and the current high participation and good performance of NRLs and EU Official Control laboratories in proficiency testing for *E. coli* and *Salmonella* spp (statutory determinands).

16. The CRL agreed to draft a proposal on proficiency testing performance assessment and follow-up for use by NRLs in supervision of Official Control laboratories. The proposal would be circulated for comment and then posted on the CRL website.

17. NRLs recognised the importance of periodic proficiency testing using matrix samples. NRLs requested that the CRL organise a further distribution of whole bivalve shellfish for enumeration of *E. coli* and detection of *Salmonella* spp in the autumn 2008.

18. Further to the above, participation in the whole bivalve shellfish proficiency test would be open to Official Control laboratories nominated by NRLs. However, the CRL would need to recover the costs for Official Control laboratory participation. The CRL would invite expressions of interest from NRLs in due course.

FRNA bacteriophage

19. NRLs recognised that the value of proficiency testing for FRNA bacteriophage was limited due to the low numbers of participants in the scheme. It was agreed that the FRNA bacteriophage PT would be discontinued.

20. However, the CRL would provide, on request, lenticulated reference material for FRNA bacteriophage together with estimated reference values, for laboratories wishing to carry out their own internal quality performance checks.

Viruses

21. NRLs noted the value of the CRL norovirus and hepatitis A proficiency testing scheme and requested that a further distribution should be offered in 2008. The CRL would include information on the reference strains used in the final report. PT samples would be in lenticule format.

22. In addition, NRLs requested that the CRL make available norovirus and hepatitis A reference material in lenticule format with supporting CRL derived reference values and strain characterisation.

23. NRLs supported the work of CEN/TC275/WG6/TAG4 'Viruses in food' on the development of a standard method for detection of norovirus and hepatitis A virus in bivalve molluscs and identified that it was now important to generate data on virus prevalence in bivalve mollusc production areas and end-products to inform risk management options.

24. The workshop noted that action following a suspect bivalve mollusc associated norovirus illness incident, or rapid alert notification, was not formalised within the EU. Action generally performed by the Competent Authority was a local investigation of the circumstance, and a request for norovirus testing of the products causing illness (if available) and the production area. Decisions on closure/opening of production areas were generally on an ad-hoc basis (not formalised) and normally considered risk factors in addition to norovirus test results.

25. With respect to the above the Commission noted that, considering the current absence of specific EU controls on viruses in LBMs, this seemed a reasonable approach under EU Food Hygiene law (Regulation 178/2002).

26. The workshop noted the importance of applying the same sampling and testing approach to own Member State production as was applied to intra-community trade.

Vibrios

27. NRLs recognised the value of the *V. parahaemolyticus* proficiency testing and requested that the CRL organised an additional round of PT for vibrios during 2008-2009. Expressions of interest would be invited amongst the network in due course.

28. NRLs supported the work of CEN/TC275/WG6/TAG3 in the development of enumerative methods for the detection of potentially pathogenic *V. parahaemolyticus.* NRLs identified that it was now important to generate data on prevalence in bivalve molluscs across the EU to inform future decisions on Official Control. It was recommended that any such studies include methods that enable detection of pathogenicity principles.

29. NRLs resolved that there should be a dedicated vibrio session at the next workshop to assist in identifying knowledge gaps, recommend appropriate methodologies and future control strategies. It was recognised that it would be beneficial to invite additional delegates with specific expertise in this area.

Next meeting

30. Provisionally it was agreed that the next workshop of NRLs would be held in Split, Croatia. The provisional date of the next workshop would be between the 12th and 14th of May 2009.

Annex III–AGENDA OF THE 7th WORKSHOP OF NRLS FOR BACTERIOLOGICAL AND VIRAL CONTAMINATION OF BIVALVE MOLLUSCS 2008

AGENDA

7th Workshop of Microbiological NRLs, 6-8 May 2008

Venue: CRL Cefas (Weymouth Laboratory
Barrack Road
The Nothe
Weymouth
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DT4 8UB
UK
Tel. +44 (0) 1305 206600
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Day 1 - Tuesday 6 May 9:30 - 18:00

1. Welcome meeting

1.1 Welcome and introductions.

1.2 Domestic arrangements including reclaim of expenses **(WS07-01, WS07-02)**

1.3 Actions arising from the 6th workshop 2007 **(WS07-03)**

1.4 Agreement of the agenda **(WS07-04)**

1.5 Introduction by Mr Lennart Johanson- The role of DG SANCO in the CRL/NRL network.

2. Questionnaire to laboratories 2007- Round Table

2.1 CRL update 2007-2008 (CRL, Rachel)

2.2 Update on progress against issues arising from the 'Questionnaire to laboratories 2007'- ***NRLs should have available the following information as a minimum:***

- **Significant changes in bivalve mollusc production, where appropriate.**
- **Number of Official Control Laboratories (OCL) undertaking microbiological testing of bivalve molluscs.**
- **Method used by OCL for enumeration of *E. coli*.**
- **Number of OCL undertaking proficiency testing for *E. coli* in bivalve molluscs.**
- **Accreditation status of NRLs with respect to ISO TS 16649-3 and EN/ISO 6579.**
- **Accreditation status of OCL undertaking classification testing of production areas with respect to ISO TS 16649-3.**

2.3 Demonstration of the CRL website, including use of the forum.

3. Proficiency testing (PT) programmes

3.1 NRLs participation and performance in the CRL/HPA Shellfish EQA for *E. coli* and *Salmonella* (CRL, Louise).

3.2 NRLs participation and performance in the CRL whole animal distribution for *E. coli* and *Salmonella* (CRL, Rachel).

3.3 FRNA bacteriophage PT (CRL, Louise).
(future PT requirements)

3.4 National in country PT programmes.

3.5 Discussion on PT performance assessment and follow-up activities (CRL, Rachel)

4. Microbiological Methods and Monitoring

4.1 Guidance on the use of the 3x5 MPN Tables in ISO 7218 (2007) (CRL, Rachel)**(WS07-10)**

4.2 Report on the progress of the ISO 16140 validation of the impedance method for the enumeration of *E. coli* against ISO 16649-3 (NRL France, Dr Martial Catherine).

4.3 Summary of the plans for validation of the ISO 16649-2 (TBX method) (NRL Netherlands, Dr Ciska Schets)

4.4 Analytical tolerance - Regarding Regulation (EC) No1666/2006 (5) reinstatement of 10% tolerance for category B areas (CRL, Rachel)

4.5 Summary of responses from the CRL questionnaire to NRLs with respect to classification and testing of live echinoderms, tunicates and gastropods (CRL, Rachel).

4.6 Experiences of NRLs in the application of the requirements for "sanitary surveys" in Regulation (EC) 854/2004.

4.6.1 NRL Italy

4.6.2 NRL Portugal

4.6.3 NRL Ireland

4.6.4 NRL Spain

4.7 Announcement of the joint EU CRL/FDA International workshop on the application of sanitary surveys in bivalve mollusc production areas (CRL).

4.8 Community Guide to the Principles of Good Practice for the Microbiological Monitoring of Bivalve Mollusc Harvesting Areas with regard to Regulation 854/2004 **(WS07-07)**(CRL/DG SANCO).

5. Depuration

5.1 Discussion on the potential requirement for establishment of an expert working group to discuss best practice with respect to commercial depuration across Europe (see Resolution 21 2007, Resolution 17, 2006).

Day 2 - Wednesday 7 May 9:30 - 18:00

6. Viruses

6.1 Report on NRLs participation in RT25 norovirus and hepatitis A PT (CRL, Louise)

6.2 Progress of CEN/TC275/WG3/TAG4 "viruses in food" (CRL, James)

6.3 Plans for validation of CEN/TC275/WG3/TAG4 "viruses in food"- performance characterisation and interlaboratory study (CRL, Rachel)

6.4 Norovirus testing in South Korea- Regulatory requirements, methods, application and consequences (Dr Hongsik Yu, Ministry of Maritime Affairs and Fisheries, MOMAF, Korea)

6.5 Update on norovirus survey in Ireland (NRL Ireland, Dr Sinead Keaveney)

6.6 Initial observations on UK restaurant study (CRL, James)

6.7 Norovirus quantitation in oysters (NRL France, Dr Soizick LeGuyader)

6.8 Virus outbreaks (NRL Denmark, Dr Anna-Charlotte Schultz)

6.9 Virus outbreaks (NRL France, Dr Soizick LeGuyader)

6.10 Bivalve Molluscan Shellfish- managing the virus risk the way forward (NRL Ireland, Mr Bill Dore)

6.11 Discussion - national approaches to virus testing/monitoring of shellfisheries, interpretation of test results and follow-up procedures.

7. Vibrios

7.1 Report on NRLs participation in RT22 *V. parahaemolyticus* PT (CRL, Louise).

7.2 *Vibrio* spp. research (NRL Netherlands).

7.3 International perspective on management of *V. parahaemolyticus* in bivalve shellfish, activities in the EU (CRL, Rachel)

7.4 Discussion on future directions for *Vibrio* spp. research, identifying knowledge gaps and control strategy.

Day 3 - Thursday 8 Day 9.00 - 11.00

8 Agreement of Workshop resolutions

9 Any other business

10 Date and venue for next meeting

Meeting close

- 11 am Surveying Molluscs (SUMO) (Sébastien Chantereau-CNC/EMPA)

Dr Chantereau will give a short presentation on the SUMO project proposal. Please note that Dr Chantereau will not be attending the NRLs workshop.

European Community Reference for monitoring bacteriological and contamination of bivalve molluscs

International Workshop on the Application of Sanitary Surveys
16-19th September 2008
Jointly presented by the CRL and USFDA

Location:
CRL for bacteriological and viral contamination of bivalve molluscs,
CEFAS,
Weymouth,
DT4 8UB.

Final programme

	Day 1: Joint CRL/FDA workshop on application of sanitary surveys **Tuesday, September 16th 8:30am -5:30pm**	**Speaker**
	Session 1. Introductory sessions. **Welcome to the CRL and introduction to the aims of workshop (8:30am)**	Rachel Rangdale (CRL)
1.	Introduction to the requirements of NSSP elements of shellfish safety and concepts of growing area classification (9:00-9:30am). • The National Shellfish Sanitation Program (NSSP) • Basic Concepts of Shellfish Area Classification	Paul DiStefano (US FDA)
2.	Introduction to Microbiological Monitoring and Sanitary Surveys in the European Union. (9:30-10:00am)	Ron Lee (EU CRL)
3.	NSSP approach (10:00-10:30 am) • Contaminant and Pollutant Hazards • Sources of Contamination • Wastewater Collection and Treatment Systems	William Burkhardt, Peter Koufopoulos Greg Goblick (US FDA)

	Coffee	To 10:45am
4.	EU Approach to Sanitary Surveys (10:45-11:15am)	Michelle Price-Hayward (CRL)
5.	NSSP Approach (11:15am-12:00pm) • Requirements for Sanitary Survey Reports • Bacteriological Surveys • Shoreline Surveys • Hydrographic Studies	 Kevin Calci Kevin Calci Kevin Calci Greg Goblick (US FDA)
6.	Hydrography and the Assessment of Bivalve Molluscs Production Areas (12:00-12:30pm)	John Aldridge (CEFAS LWT)
7.	**NSSP Approach (12:30-1:00pm)** • **Pollution Source Assessments** • Management Strategies • Wastewater Treatment Plant- Management Scenarios • Wastewater Treatment, Viruses and Shellfish	 Greg Goblick Peter Koufopoulos Greg Goblick William Burkhardt (US FDA)
	Lunch	To 2:00pm
8.	NSSP approach (2:00-3:25pm) • Management Strategies • Wastewater Treatment Plant- Management Scenarios • Wastewater Treatment, Viruses and Shellfish • Restricted Growing Areas and Shellfish Depuration and Relaying	 Peter Koufopoulos Greg Goblick William Burkhardt William Burkhardt (US FDA)
	Tea	To 3:45pm
9.	EU approach - Interpretation of monitoring programme data for the classification of harvesting areas (3:45 –4:30pm)	Andrew Younger CRL Martial Catherine Ifremer, France
10.	EU approach - Management and remediation strategies- Current and Potential EU Approach (4:30-5:00pm)	Bill Doré Marine Institute, Ireland Simon Kershaw CRL
11.	Fleet Lagoon- An introduction to the study area and background to the practical work (5:00-5:30pm)	Carlos Campos CRL

Note. All times are indicative

	Day 2: Wednesday, September 17th **Session 2: Shoreline Survey** 10:00-18:00	
13.	Shoreline survey (10:00-18:00)- attendees will be taken to a local bivalve shellfishery (The Fleet) To include rhodamine tracer study, on and off-shore survey (demonstration activity) **A packed lunch will be provided**	All

	Day 3: Thursday, September 18th Session 2 cont: **Shoreline survey** (8:30 – 10.30 am)	
14.	Wash-up session 1:Tracer study feedback (8:30-9:30am)	Led by US FDA
15.	Wash-up session 2: Shoreline survey (9:30-10:30am)	Led by CRL
	Coffee	To 11am
	Day 3: Thursday, September 18th **Session 3: Case studies** (11am-6pm)	
16.	Case study 1: EU: Islay: Loch Gruinart (11am-11:40am) • Application of Sanitary Surveys in Scotland • Introduction to Islay: Loch Gruinart • Overview of Data and Assessment Islay: Loch Gruinart • Introduction to EU Workshop Session	Lorna Murray (FSAS) Michelle Price-Haywood (CRL) Lorna Murray (FSAS)
17.	Case study 1 EU: Islay: Loch Gruinart: break out groups (11:45am-1:30pm)	Led by CRL
	Lunch	To 2pm
18.	Case study 1 EU: Islay: Loch Gruinart (2 - 2:50pm)- reports from break out groups	Led by CRL
	Tea	To 3pm
20.	Case study 2: US (3-6pm) • Shellfish Accumulation/Effluent Dilution Study- Mobile Bay	Led by USFDA

	2008 • Dye Study of Tributary- Demonstration Project Korea 2008 • Determining Impact from Proposed Discharge- Chinocoteague Island, Virginia 2006 • Case Studies	William Burkhardt

Note. All times are indicative

	Day 4: Friday, September 19th **Session 4: Other perspectives on sanitary surveys**	
18.	Australian perspective (9:00- 9:45pm)	Ken Lee (PIRES)
19.	Asian perspective (9:45-10.30am)	HongSik Yu (Korea)
	Coffee	*To 10.45 am*
20.	Sanitary Surveys in Bivalve Molluscs Production Areas: The Italian Experience (10.45-11:30am)	Giuseppe Arcangeli and Luigi Lanni (Italy)
21.	Canadian perspective (11.30am-12pm)	CFIA John White
22.	Chinese perspective (12-12:20 pm)	Chen Jing AQSIQ
	Lunch	To 1:15pm

	Day 4: Friday, September 19th **Session 5: Discussions and recommendations** (1:15 pm-4 pm)	
22.	Comparing International and National approaches (1:15-2pm)	All
23.	Best practice approaches and development of new initiatives for the future (2-3:30pm)	All
	Tea	To 3:30pm
24.	Workshop recommendations, workshop close 4pm	All

European Community Reference laboratory
for monitoring bacteriological and
contamination of bivalve molluscs

Conclusions and Recommendations of the 1st International Workshop on the Application of Sanitary Surveys

1. The workshop recognised that the discussions and exchanges of information had been valuable but identified that the initiatives needed to be developed by means of further meetings and, where appropriate, the formation of working groups.

2. The workshop noted that it was important to demonstrate the equivalency of the approaches to reviewing and updating sanitary surveys, but that it may not be necessary to conduct a new sanitary survey every twelve years as required under the US FDA NSSP, provided the approach and frequency of review was demonstrably accurate and protective of public health.

3. The workshop noted that if national approaches to sanitary surveys were consistent amongst countries, fit for purpose and demonstrably equivalent in terms of public health protection outcomes, movement towards equivalency in other areas such as measurement of faecal contamination indicators in flesh versus water could be progressed.

4. Considering the requirement of 1000:1 dilution for WWTP effluents, the FDA noted that an explanatory document outlining the history and setting out the scientific justification underpinning the dilution factor was in preparation. The document would be presented at ISSC in 2009 and would be circulated to the workshop on completion.

5. The workshop noted that it was important to recognise and understand the dynamic nature and variety of WWTPs, and to understand that their efficacy with respect to removal of faecal indicators and viral pathogens may vary considerably.

6. It was recognised that it would be useful to elaborate best practice guidance to support assessments of the effectiveness and the relevant operational parameters of WWTPs with respect to interpretation of data for inclusion in sanitary surveys.

7. With respect to the above it was recognised that acquisition of additional data on virus removal (norovirus and hepatitis A) during wastewater treatment was desirable.

8. It was suggested that provided sanitary surveys were undertaken properly and fully as part of a shellfish sanitation programme they offered good public health protection, and that generally where information was available substantiated viral outbreaks associated with shellfish consumption were as a result of failures in the programme to comply with programme requirements.

9. The workshop agreed that prevention of shellfish associated illness was of primary importance and that sanitary surveys could be a significant tool to inform proactive procedures to protect public health.

10. It was recognised that management and remediation actions should be reflective of the nature of the problem e.g. based on evidence of bivalve shellfish associated illness of viral aetiology extended closures may be required compared with bacterial or toxin events.

11. It was recognised that it was problematic in terms of resources for countries wishing to export bivalve shellfish to the EU and US to operate dual programmes. The workshop recommended that an approach should be made to Codex and FAO/WHO setting out the opinion of the workshop with respect to the comparability of approaches with a view to development of international guidelines on equivalency.

12. Further to the above it was recognised that in order to measure the success of the approaches to shellfish sanitation programmes and thus enable demonstration of their equivalency, it was desirable to identify suitable arbiters, and that whilst these may include public health outcomes, considerations should be given to other performance indices.

13. The workshop recognised the importance of enforcement, and acknowledged that it comprised several elements (audit, policing etc), the workshop affirmed that the desired outcomes of a programme would only be delivered with effective enforcement.

14. The workshop recognised that stringent quality requirements were required to enable the inclusion of industry samples in official programmes.

15. The workshop recognised the potential value of source tracking as a useful supplementary tool for conducting sanitary surveys.

16. Further to the above the workshop supported further research in the area to develop fit-for-purpose approaches and recognised that these may need to consider temporal and geographical variability.

Annex VI -Summary of participation by NRLs and others in CRL organised proficiency testing

CRL ring trial reference number	Ring trial description	Austria	Belgium and Luxembourg	Bulgaria	Cyprus	Czech Republic	Denmark	Estonia	Finland	France	Germany	Greece	Hungary	Ireland	Italy	Latvia	Lithuania	Malta	Netherlands	Poland	Portugal	Romania	Slovakia	Slovenia	Spain	Sweden	United Kingdom	Croatia	Turkey	Iceland	Norway	United States	New Zealand	South Korea	Chile	Hong Kong	Canada
RT 28	*E.coli/Salmonella* whole animal 2007	✓	x	✓	x	x	✓[a]	x	✓	✓	✓	✓	✓	✓[a]	✓	✓	✓	x	✓[a]	✓	✓	✓	✓	✓	✓	✓[a]	✓[a]	✓[c]	x	x	✓	x	x	✓[c]	x	x	x
RT 25	Norovirus/Hepatitis A 2008	✓	✓	x	x	x	✓	✓[d]	x	✓[c]	✓[c]	x	✓[d]	✓	✓	x	x	x	✓[c]	x	✓	✓	✓	✓	✓	✓	✓	x	x	x	✓	✓	✓	✓[c]	✓[c]	✓	✓
RT 22	*Vibrio parahaemolyticus* 2008	✓	✓	✓	x	x	x	✓	x	x	✓	✓	✓	x	✓	✓	x	x	✓	✓	✓	x	✓	✓	x	x	✓	✓	✓	✓	✓	✓	x	x	x	x	✓
RT 30	*E. coli/Salmonella* EQA 2008	✓	✓	x	x	✓	✓	✓	✓	✓	✓	✓	x	✓	✓	x	✓	x	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	x	✓	[e]	[e]	[e]	[e]	[e]	[e]

[a] additional Official Control laboratories participated in the ring trial
[b] designated laboratory(ies) carried out PT on behalf of NRL
[c] additional laboratories participated in the ring trial
[d] no results returned
[e] participation or performance in CRL/HPA scheme not examined

European Community Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

Report on the whole animal bivalve shellfish ring trial:

Enumeration of *Escherichia coli* and the detection of S*almonella* spp..

CRL ring trial reference: RT 28 (*E. coli/Salmonella* 2008)

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Contents

Tables

Figures

Appendix

1.0 Introduction

In November 2008 the CRL organised a distribution of naturally contaminated common mussels (*Mytilus edulis*) for enumeration of *E. coli* and detection of *Salmonella* spp.

2.0 Proficiency testing sample

2.1 Sample preparation and distribution

One batch, consisting of approximately 1500 *M. edulis*, was collected from a UK commercial producer. On arrival at the Cefas laboratory sub-samples of approximately 35 mussels were selected at random and placed in clean plastic sample bags containing a numbered temperature logger (Thermotrack, Progress Plus). Individual samples were placed in 10 litre Biotherm thermal control units and packaged according to Cefas procedure for use of 10L Biotherm boxes and IATA regulations. Fifty-five samples were distributed under refrigeration conditions by CitySprint courier services on 24th November 2008. The remaining mussels were stored at 3±2˚C for reference testing. On receipt, participants were requested to analyse the material immediately and to return the temperature logger to the CRL.

2.2 Sample temperature

Participants were asked to manually record the internal air and sample temperature on arrival and return the temperature logger. The temperature range (as determined from downloaded loggers), and participants' temperature measurements are given in Appendix I.

3.0 Results

3.1 Confidentiality of results

Each laboratory was provided with a personal identification number to preserve anonymity.

3.2 Reference results

Twelve sub-samples were analysed using the CRL standard methods (ISO 16649-3 and ISO 6579). The reference results are summarised in Table 1 and included, for *E. coli*, in Figure 1.

Table 1: The *E.coli* range and *Salmonella* spp. presence / absence from the reference results

Sample description	*E. coli* MPN/100g	*Salmonella spp..* Present in 25g	Absent in 25g
M. edulis	1.7×10^3 – $>1.8 \times 10^4$	4 from 12	8 from 12

3.3 Analysis of results

Participants duplicate *E. coli* and *Salmonella* results are given in Table 2. The median and upper and lower limits (±3 standard deviations (SD) and ±5 SD) were calculated from participants' results. SD calculations were based on the expected inherent variability of the five tube MPN method which is 0.26 $\log_{10}$. The results chart was compiled using $\log_{10}$ transformed MPN values (Figure 1). Reference results were omitted from this calculation. Performance assessment was carried out according to the procedures described in the CRL/HPA EQA shellfish scheme, with minor modifications to reflect replicate analyses of a single sample (Appendix II). The participants' and reference results median and SD and the expected range for *E. coli* estimated from participants' results are given in Tables 3 and 4. Scoring for detection of *Salmonella* spp. was not undertaken.

Table 2: Results reported by participants for *E.coli* and *Salmonella* spp..

Lab ID	*E.coli* MPN/100g			*Salmonella* spp. in 25g	
	Replicate 1	Replicate 2	Score	Replicate 1	Replicate 2
3	1700	2400	12	absent	absent
7	1300	790	12	absent	absent
9	1100	1400	12	absent	absent
10	3500	1300	12	absent	absent
11	1100	1100	12	absent	absent
12	750	2000	12	absent	absent
13	5400	2400	12	absent	absent
19	1100	3500	12	absent	absent
21	90	310	9	absent	absent
22	1100	1100	12	absent	absent
23	310	950	12	absent	absent
26	790	490	12	absent	absent
27	750	2400	12	absent	absent
30	750	1700	12	absent	absent
32	750	1300	12	absent	absent
33	1300	2400	12	absent	absent
35	1100	1700	12	absent	absent
36	2400	1300	12	absent	absent
39	1400	2100	12	absent	absent
41	1300	2400	12	absent	absent
42	5400	3500	12	absent	absent
43	1400	1300	12	absent	absent
44	330	490	12	absent	absent
47	2100	330	12	absent	absent
56	310	750	12	NE	NE
58	1300	700	12	absent	present
61	3500	1300	12	absent	absent
63	700	750	12	absent	absent
64	790	4600	12	NE	NE
68	490	330	12	absent	absent
70	790	790	12	absent	absent
77	2400	2200	12	absent	absent
79	1700	1700	12	absent	absent
85	5400	750	12	absent	absent
86	1300	2000	12	absent	absent
88	2400	2400	12	absent	absent
90	1300	1700	12	absent	absent
96	700	230	12	absent	absent
101	1700	2400	12	absent	absent
103	1300	490	12	absent	absent
108	1700	1300	12	absent	absent
109	310	500	12	absent	absent
112	NR	NR	2	NR	NR
117	3500	2400	12	absent	absent
120	3500	1100	12	absent	absent
125	3500	2200	12	absent	absent
126	3500	1100	12	absent	absent
131	500	500	12	NE	NE

Lab ID	*E.coli* MPN/100g			*Salmonella* spp. in 25g	
	Replicate 1	Replicate 2	Score	Replicate 1	Replicate 2
135	2200	2200	12	absent	absent
139	5400	3500	12	absent	absent
142	1300	1300	12	absent	absent
144	500	500	12	absent	absent
148	1100	1100	12	absent	absent
149	1300	1100	12	absent	absent
150	5400	5400	12	absent	absent

NE - Not examined
NR - Not reported

3.4 Summary participation statistics for RT 28

Total participants reporting duplicate results for *E. coli* MPN	54
Participants reporting single MPN	0
Participants reporting MPN results within the expected range[1]	53
Participants reporting MPN results outside the expected range for one replicate	1
Participants not returning results	1

[1]expected range = participants' median ± theoretical 3SD

Table 3. Participants and reference results for *E. coli* MPN – replicates combined

	Median MPN/100g	Standard deviation
Reference results	$2.6 \times 10^{4*}$	1.5×10^{4}
Participants results	1.3×10^{3}	1.3×10^{3}

* censored results were allocated a nominal score of 3.6×10^{4}

Table 4. Expected range for *E.coli* obtained from participants' results

	Expected range for *E. coli* MPN/100g (median ±3 SD)
Participants results	$2.2 \times 10^{2} – 7.8 \times 10^{3}$

4. Conclusion and recommendations

4.1 General comments

Fifty-four laboratories (20 NRLs and 34 in country Official Control laboratories) returned results for the distribution. Fifty-six percent of samples arrived within 24 hr of dispatch. Thirty-eight laboratories analysed the samples on the day of arrival. Of the remaining 16 laboratories, 15 analysed on the following day (i.e. within 48-72 hr of dispatch).

4.2 *E. coli* analysis- participants' results

Fifty-three laboratories returned replicate results between ±3 SD of the participants' median. Laboratory 21 returned a single replicate between 3 and 5 SD of the participants' median. Laboratory 112 did not examine the sample within the specified timeframe. The laboratory indicated that the sample had arrived too late for analysis to be carried out on the day of arrival. On request of the CRL the courier (CitySprint) provided records indicating that the delivery time at the institute was 14:20. It is recommended that laboratory 112 examine their internal quality procedures for receipt of samples.

Laboratories 12, 47, 64, 86 and 144 returned MPN results that were not consistent with the CRL guidance on interpretation of 5x3 MPN tables. For CRL guidance on the interpretation of MPN tube combinations see Appendix III. Laboratory 39 gave a tube combination (5,3,4) which would, using CRL guidance on use of MPN tables, be considered void. The laboratory reported an MPN value of 2100 which was within the expected range.

Fifty-three laboratories from the fifty-four returning results reported the use of the *E. coli* reference method (ISO TS 16649-3) or Donovan *et al* 1998. Laboratory 64 reported the use of NMKL 96 for enumeration of *E. coli.* This method has not been formally validated according to ISO 16140 for bivalve shellfish.

4.2 *E. coli* enumeration- reference results

Reference results generated by replicate sample testing by the NRL indicated a high level of between sample variability, with 6 sub-samples returning *E. coli* MPN/100g of >1.8 x10^4 (range 1.7 x 10^3 –>1.8 x 10^4). For estimation of the reference result median and graphical representation of data points censored values were allocated a nominal score of 3.6 x 10^4.This high level of variability was not observed in participant's results. The reasons for this sample to sample variation were not clear, however on investigation it was identified that all reference sub-samples were derived from one of three large boxes containing the total consignment, whereas participants' sub-samples were extracted from the two remaining boxes. The mussels were harvested by the producer and collected by a commercial courier from the site already boxed. It is speculated that mussels from the third box (reference material) were sampled from a different lateral or longitudinal position across the bed that had been exposed to different levels of faecal contamination. This degree of between sample variation is substantially outside the observed measurement uncertainty of a 5-tube, 3-dilution MPN assay for live bivalve shellfish (Anon 2003), thus it is suggested that unusual environmental factors influenced sample content on this occasion.

The CRL will produce written procedures for randomisation of ring trial sub-samples to reduce the potential for reoccurrence of this anomaly.

4.3 *Salmonella* spp. analysis and methodology

Only one laboratory detected *Salmonella* spp in a single replicate.

Seventy-six percent of laboratories used the EU specified reference method for *Salmonella* spp (ISO 6579). Three laboratories referenced NMKL 71: *Salmonella* detection in foods. Two laboratories referenced Vidas and BAX (PCR method) to detect for *Salmonella* analysis. These methods have not been formally validated according to ISO 16140 for bivalve shellfish.

4.4 *Salmonella* spp. analysis- reference results

Salmonella spp. was detected in a third of the reference samples. At low levels *Salmonella* may have been absent in a proportion of samples and potentially below or at the limit of detection of the assay in others. Previous studies at the CRL have estimated that the limit of detection of ISO 6579 in seeded bivalve shellfish matrix is 4CFU/25g. However, given the elevated *E. coli* levels in a sub-population of the mussel samples, it is considered likely that detection of *Salmonella* spp. in the reference results was associated with exposure to high levels of faecal contamination. All 4 *Salmonella* spp. positive reference samples returned *E. coli* MPN/100g >1.8 x 10^4.

4.3 Transit time and temperature

The maximum internal temperature of samples during transit as determined by the automatic temperature loggers did not exceed the maximum receipting of 10°C as recommended by the CRL (Anon 2007).

5. References

Anon 2004 ISO/TS 16649-3:2004. 'Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of β-glucuronidase-positive *Escherichia coli* Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide'.

Anon 2003 Report (WD SANCO/58/2003) upon Measurement uncertainties in microbiological analyses- with special reference to the uncertainties linked to the proposed microbiological criteria in the document SANCO/4198/2001, rev.5 .

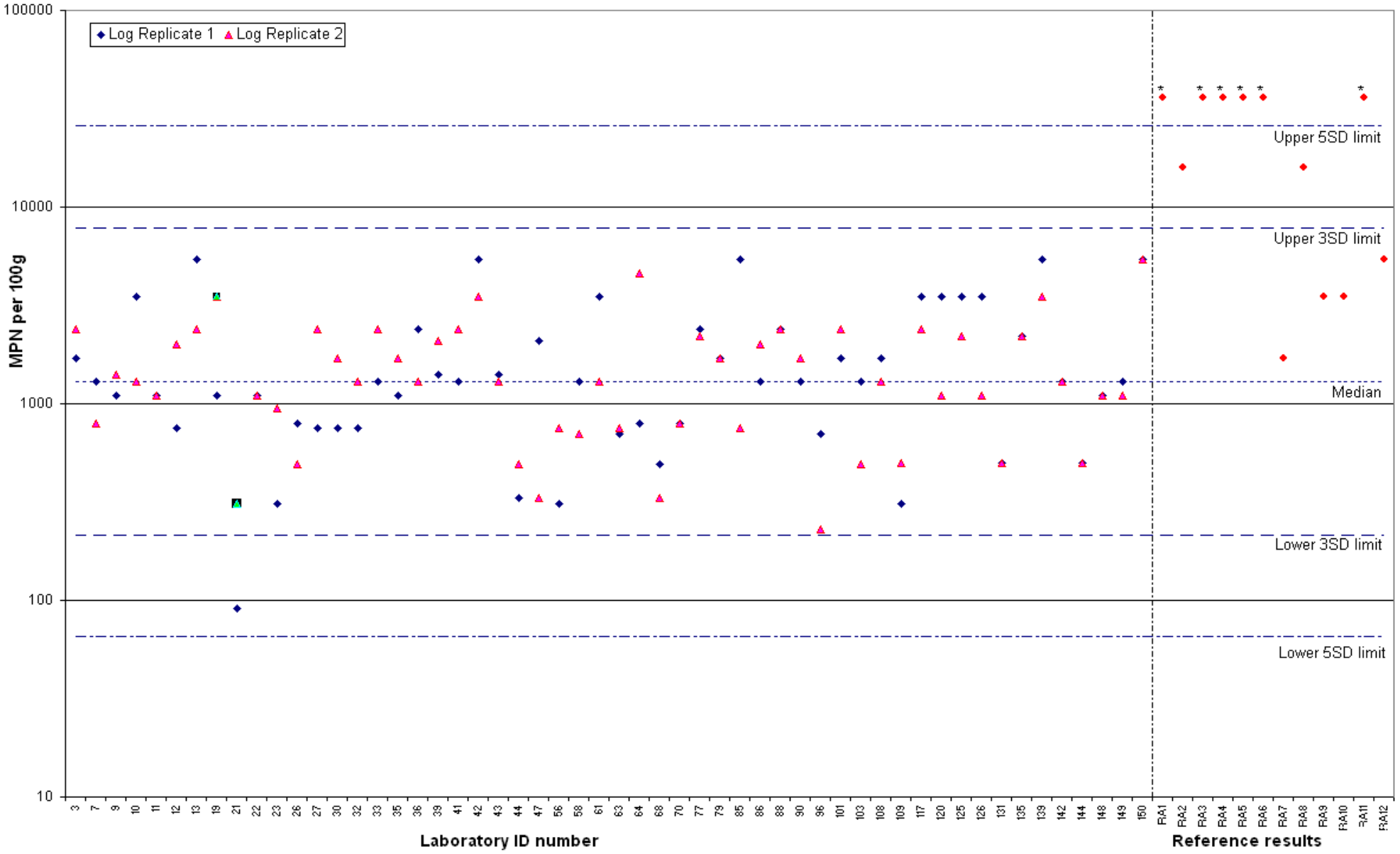
Anon. 2002. ISO TS 6579-2002. Microbiology of food and animal feeding stuffs – Horizontal method for the detection of Salmonella spp. ISO Copyright Office, Case Postale 56, CH-12111, Geneva 20, Switzerland.

Anon 2007. Microbiological monitoring of bivalve mollusc harvesting areas, a guide to good practice: technical application. CRL publication, issue 3, February 2007.

Donovan TJ, Gallacher S, Andrews NJ, Greenwood MH, Graham J, Russel JE, Roberts D, Lee R.1998. 'Modification of the standard method used in the united kingdom for counting *Escherichia coli* in live bivalve molluscs'. Communicable disease and public health 1: 188-96.

Figure 1: Participants duplicate results for *E.coli* (MPN) on whole animal bivalve shellfish

Key - * *E.coli* reference results were given value of 36,000 as results reported were >18,000.



Appendix I: Sample temperatures

All samples were dispatched on the 24th November 2008.

Country	Time of arrival	Date of arrival	Temp. logger (°C)[1]	Internal air (°C)[2]	Sample (°C)[2]	Storage (°C)[2]	Date analysed
Austria	09:30	26.11.08	<4.0	5.3	2.3	2 – 6	26.11.08
Bulgaria	14:40	25.11.08	<9.0	6.1	3.2	4	26.11.08
Croatia	12:15	27.11.08	<4.0	3.2	3.3	4	27.11.08
	11:24	27.11.08	<6.0	7.2	2.9	-	27.11.08
Denmark	13:30	26.11.08	<8.0	6.6	4.6	5	27.11.08
	11:15	26.11.08	<8.5	-	3	3	26.11.08
	11:20	26.11.08	<9.5	2.6	2.5	-	26.11.08
Finland	15:00	25.11.08	<5.0	-	-	5	26.11.08
France	09:30	26.11.08	<4.0	8.2	2.2	4.2	26.11.08
Germany	09:35	26.11.08	-	4	3	-	26.11.08
Greece	12:35	27.11.08	<8.0	3.4	3.2	5	27.11.08
Hungary	11:30	25.11.08	<3.5	5.5	3.2	3.5	26.11.08
Ireland	09:30	25.11.08	<5.0	6.3	3.1	-	25.11.08
	12:05	26.11.08	-	5.2	4.3	3.1	26.11.08
	11:30	26.11.08	-	4.5	4.2	3.6	26.11.08
	14:30	26.11.08	-	-	4	-	26.11.08
	15:00	25.11.08	<9.0	5	5	3	26.11.08
	10:00	26.11.08	<8.5	9.7	3.4	4	26.11.08
Italy	-	27.11.08	<7.5	3	1.5	4	27.11.08
Korea	18:00	27.11.08	<8.0	4	3.9	-	27.11.08
	15:40	28.11.08	<8.5	7.3	6.8	-	28.11.08
	12:57	27.11.08	<6.5	6	3.7	2.7	27.11.08
Latvia	15:30	25.11.08	<6.0	5.4	5	5	26.11.08
Lithuania	15:50	25.11.08	<5.0	0.4	4	4	26.11.08
Netherlands	11:00	27.11.08	<5.0	-	-	4	28.11.08
	10:30	26.11.08	<3.5	-	3.9	4	27.11.08
Norway	15:00	25.11.08	<2.5	-	-	-17	26.11.08
Poland	14:35	26.11.08	<6.5	7.5	6.2	2 – 8	27.11.08
Portugal	10:50	25.11.08	-	8	4	2 – 7	25.11.08
Romania	17:20	26.11.08	<6.0	3.2	2	4	27.11.08
Slovakia	11:00	26.11.08	-	-	-	3	27.11.08
Slovenia	12:00	25.11.08	<6.0	5	5	-	25.11.08
Spain	13:35	26.11.08	<9.5	5	4	1 – 3	27.11.08
Sweden	13:30	25.11.08	<8.0	-	3	5	25.11.08
	11:30	25.11.08	<6.5	3.8	1.6	4.2	25.11.08
UK		25.11.08	<6.0	-	-	2 - 5	25.11.08
	10:15	25.11.08	<3.0	-	3.7	4.1	25.11.08
	12:05	25.11.08	<4.5	2.9	0.8	4	25.11.08
	09:15	25.11.08	<7.0	-	3.6	0 - 5	25.11.08
	09:10	25.11.05	<9.5	6.5	3.5	-	25.11.08
	11:40	25.11.08	<4.0	1.8	3.4	4.5	25.11.08
	09:30	25.11.08	<5.0	10.8	3.8	-	25.11.08
	11:05	25.11.08	<6.5	2.2	2.5	5.6	25.11.08
	09:15	25.11.08	<6.0	5.8	4.4	4.2	25.11.08

	08:24	25.11.08	<2.0	6.3	3.8	4.9	25.11.08
	10:45	25.11.08	<7.5	3.7	-	4.6	25.11.08
	14:20	25.11.08	-	-	-	-	-
	09:00	25.11.08	<4.5	1.5	0.9	1.5	25.11.08
	09:09	25.11.08	<6.5	3.62	3.62	3	25.11.08
	10:28	26.11.08	<5.0	7.9	2.9	4.6	26.11.08
	09:30	25.11.08	-	7.7	7.5	4	8.12.08
	16:00	25.11.08	<9.5	9.1	4	-	25.11.08
	12:45	25.11.08	-	3.2	4.5	2.6	26.11.08
	11:50	25.11.08	<2.0	3.3	2.6	3.7	25.11.08
	16:15	25.11.08	<3.0	4.7	2.9	3.5	25.11.08

[1] temperature indicated by sample temperature recorder
[2] temperature recordings by participants.

Appendix II: *E.coli* MPN scoring

Result	Points allocated
Return of results	2
All replicate MPN results within the expected range	10
Or	
One replicate MPN result reported is outside the expected range and falls between the median ±3SD and median ±5SD value	7
Or	
Both replicate MPN results are outside the expected range and fall between the median ±3SD and median ±5SD value	4
Or	
One replicate MPN result reported is outside the median ±5SD value	5
Or	
Both replicate MPN results reported is outside the median ±5SD value	0
Or	
Single MPN result reported only	5
Or	
Tube combination inconsistent with MPN reported (one replicate)	7
Or	
Tube combination inconsistent with MPN reported (both replicates)	5
Or	
Sample not examined or results returned late - no explanation received	0
Or	
High censored result (e.g. MPN = >18000 per 100g)	Score not allocated

Appendix III: Calculation of *E.coli* Most Probable Number (MPN) and reporting

Cefas Standard Operating Procedure – Enumeration of *Escherichia coli* in molluscan shellfish 6. www.crlcefas.org. Adapted from: ISO 7218:2007.

To calculate the most probable number (MPN), record the number of TBGA plate positives for each dilution. This gives a three figure tube combination number, which is used to calculate the MPN. MPN tube combinations fall into one of four categories. 95% of observed tube combinations fall in to category 1 with 4%, 0.9% and 0.1% in categories 2, 3 and 0 respectively. Both the category and MPN result can be determined from the MPN table (see Appendix 2) as follows:

- For dilutions of neat, 10–1 and 10–2 use MPN Table 1.
- For dilutions of 10–1, 10–2 and 10–3 use MPN Table 2.
- For dilutions of 10–2, 10–3 and 10–4 use MPN Table 3.
- For greater dilutions use MPN Table 3 and multiply the result by the extra number of dilution factors.

Where more than three dilutions have been tested for a sample, select the tube combination as stated in the following rules:

1.Select the combination of three consecutive dilutions having a category 1 profile to obtain the MPN index. If more than one combination having a category 1 profile is obtained, use the one with the highest number of positive tubes.

2. If no combination having a category 1 profile is available, use the one having a category 2 profile. If more than one combination having a category 2 profile is obtained, use the one with the highest number of positive tubes.

Results should be reported as the most probable number per 100g of shellfish. Negative samples should be reported as MPN <20/100g. Where the MPN tube combination is not given in the relevant table, the result should be reported as ‘Void’.

Note: The 5-tube 3-dilution MPN table given in ISO 7218:2007 includes all category 1 and category 2 combinations, and some (but not all) category 3 combinations. A note is included in the standard that: “Before starting testing, it should be decided which category will be acceptable, that is, only 1, 1 and 2 or even 1, 2 and 3. When the decision to be taken on the basis of the result is of great importance, only category 1, or at most 1 and 2, results should be accepted. Category 0 results should be considered with great suspicion”. Given that the NRL generic SOP will be referred to by official control laboratories, all of the category 3 combinations have been omitted from the version of the tables presented here.

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European Community Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

Report on the Norovirus/Hepatitis A Ring Trial, 2008

CRL ring trial reference: RT 25 (NoV/HAV 2008)

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Appendix

1. Introduction

Regulation (EC) No 882/2004 designates the Centre for Environment, Fisheries and Aquaculture Science at Weymouth U.K. as the Community Reference Laboratory (CRL) for monitoring the viral and bacteriological contamination of bivalve molluscs. Under Article 32 the CRL laboratory is responsible for organising comparative testing by National Reference Laboratories (NRLs).

At the 6th annual workshop of NRLs Galway 2007, several resolutions were agreed relating to proficiency testing for statutory determinants including:

> **Resolution 26.** NRLs requested that the CRL organise a further ring trial for norovirus (NoV) and hepatitis A virus (HAV) in laboratory constructed samples in lenticule format, and that the distribution would include low concentration samples.

2. Proficiency testing samples

2.1 Sample preparation

Samples were comprised of laboratory constructed lenticules (RT 25 L1 to RT 25 L6). Lenticules were constructed following the methods of Codd *et al* (1998) with minor modifications. In brief, dilutions of NoV genogroup I (GI.5 capsid type; 98.6% sequence homology to Appalachicola Bay strain (AF414406)) and genogroup II (GII.4 capsid type; 99.3% sequence homology to Monastir strain (EU650225)) faecal material, and laboratory cultured HAV (strain HM175/43c grown in FRhK cells) were prepared in phosphate buffered saline (PBS) and added at a 1:5 ratio to lenticulating fluid. The inoculated lenticulating fluid was aliquoted onto parafilm in 25µl volumes and placed in a desiccating chamber at 4±2˚C for 1 week. After 1 week Lenticules were transferred to -20±5˚C prior to analysis using the CRL standard method.

2.2 Distribution of samples

The NoV/HAV ring trial 2008 was designated as RT 25. Samples were packaged according to IATA regulations, UN3373 as biological samples, division 6.2 under the packing instruction code 650. All participating laboratories received a single box comprising of a sealed plastic bag containing Lenticules RT 25 - L1 to RT 25 - L6. Samples were distributed by CitySprint on the week commencing the 25th February 2008. On receipt, participants were requested to store the samples at -20±5˚C prior to analysis during the week commencing the 10th March 2008.

2.3 Quality control at dispatch

Lenticules were tested prior to distribution to confirm the presence or absence of norovirus GI and GII and Hepatitis A virus. Analyses were undertaken using CRL standard procedures. The results are given in Table 1.

Table 1. Taqman™ expected results of RT 25 ring trial material

Sample	Norovirus GI	Norovirus GII	HAV
RT 25 – L1	-	+	-
RT 25 – L2	+	-	+
RT 25 – L3	+	+	+
RT 25 – L4	-	+	+
RT 25 – L5	+	-	-
RT 25 – L6	-	-	-

2.4 Confidentiality of results

Each laboratory participant was identified by a code to preserve anonymity.

2.5 Participation

Invitations inviting expressions of interest in the NoV/HAV ring trial were sent to all designated member state NRLs, and laboratories from EU candidate countries, European Free Trade Association member countries and selected third countries. Table 2 summarises the participation in the NoV/HAV ring trial distribution (RT 25). Material was dispatched to twenty-nine laboratories. Twenty-seven laboratories returned results for the trial (Table 4).

Table 2. Participation in the NoV/HAV ring trial distribution (RT 25)

Country	Participation in RT 25	Results returned to CRL
Austria	Yes	Yes
Belgium & Luxembourg	Yes	Yes
Bulgaria	No	-
Czech Republic	No	-
Denmark	Yes	Yes
Estonia	Yes	No
Finland	No	-
France 1	Yes	Yes
France 2	Yes	Yes
Germany 1	Yes	Yes
Germany 2	Yes	Yes
Greece	No	-
Hungary	Yes	No
Ireland	Yes	Yes
Italy	Yes	Yes
Latvia	No	-
Lithuania	No	-
Netherlands 1	Yes	Yes
Netherlands 2	Yes	Yes
Poland	No	-
Portugal	Yes	Yes
Romania	Yes	Yes
Slovakia	Yes	Yes
Slovenia	Yes	Yes
Spain	Yes	Yes
Sweden	Yes	Yes
United Kingdom	Yes	Yes
Candidate country		
Croatia	No	-
EFTA		
Iceland	No	-
Norway	Yes	Yes
Third Country		
Canada	Yes	Yes
Chile 1	Yes	Yes
Chile 2	Yes	Yes
Hong Kong	Yes	Yes
New Zealand	Yes	Yes
South Korea 1	Yes	Yes

Third Country	Participation in RT 25	Results returned to CRL
South Korea 2	Yes	Yes
United States	Yes	Yes

2.6 Reference results

Reference analyses were performed by the CRL on lenticules stored at -20±5˚C. Six randomly selected lenticules from each distribution were analysed for all viral determinants on 2 separate occasions using the CRL standard method. Real-time reverse transcription PCR (TaqMan™) analyses were performed in triplicate on viral RNA. The quantitative results of CRL reference testing are presented in Appendix IV.

3 Results

3.1 Analysis of results

Twenty-seven laboratories returned results. Two laboratories did not return results for NoV and two other laboratories did not return results for HAV. One laboratory used a non-discriminatory method for detection of NoV GI and GII, in addition to genogroup-specific methods. Results for those participants returning Ct values are shown in Appendix III. Participants' results (shown in Table 4) were assessed as percentage relative sensitivity, specificity and accuracy for each determinant according to the following calculations:

Percentage relative sensitivity:

$$\text{Relative sensitivity (SE)} = \frac{TP}{(TP+FN)} \times 100\%$$

Percentage relative specificity:

$$\text{Relative specificity (SP)} = \frac{TN}{(TN+FP)} \times 100\%$$

Percentage relative accuracy:

$$\text{Relative accuracy (AC)} = \frac{TP+TN}{N} \times 100\%$$

Where TP = true positives
FN= false negatives
FP = false positives
TN= true negatives
N = total number of tests

Note: Participants' results were expressed as percentage concordance with intended results generated by the CRL. In this assessment presence/absence data was used and no consideration of quantitative measurements (Ct values) was made.

Table 3. Participants' results for all lenticules (L1 - L6)

Lab ID number	GI			GII			HAV		
	SE	SP	AC	SE	SP	AC	SE	SP	AC
2	100	100	100	100	100	100	100	100	100
3	83	100	92	100	100	100	100	100	100
9	100	67	83	67	67	67	100	100	100
10	100	100	100	100	83	92	100	100	100
11	100	100	100	100	100	100	100	100	100
13	NE	NE	NE	NE	NE	NE	NE	NE	NE
14	NE	NE	NE	NE	NE	NE	100	100	100
15	0	100	50	67	100	83	83	100	92
17	67	100	83	0	100	50	100	100	100
19	100	100	100	100	100	100	100	100	100
21	100	100	100	100	100	100	100	100	100
24	100	100	100	100	100	100	100	100	100
25	100	100	100	100	100	100	100	100	100
27	67	100	83	50	100	75	33	100	67
32	0	100	40	100	100	100	100	100	100
33	100	100	100	100	100	100	100	100	100
35	67	100	83	0	100	50	100	100	100
37	100	100	100	100	100	100	100	100	100
39	67	100	83	0	100	50	100	100	100
41	50	100	75	100	100	100	100	100	100
43	NE	NE	NE	NE	NE	NE	NE	NE	NE
47	NE	NE	NE	NE	NE	NE	100	100	100
48	100	100	100	100	100	100	100	100	100
55	0	100	50	0	100	50	83	83	83
89	100	100	100	100	83	92	NE	NE	NE
94	0	100	50	100	100	100	100	100	100
113	0	100	50	0	100	50	NE	NE	NE
133	100	100	100	83	100	92	83	100	92
146	83	100	92	100	100	100	100	100	100

NE - Not examined
Lab 13 - Did not take part in the ring trial.
Lab 14 and Lab 47 - Did not test for NoV.
Lab 17 - High Ct values detected in GII positive controls for real-time PCR, results not reported and insufficient time to repeat.
Lab 32 - Did not test lenticule 1 due to problems during extraction, also had problems with dissolving pellet in lenticule 6.
Lab 39 - Reported NoV (non-specific) results in addition to genogroup-specific results. NoV SE – 60%, NoV SP – 100% and NoV AC – 66%.
Lab 43 - Problems with primers for NoV method. Hepatitis A was not tested.
Lab 89 and Lab 113 - Did not test for HAV.

4 Discussion

- Laboratory constructed lenticules produced by the CRL provided a stable, homogeneous reference material for the ring trial that could be transported at ambient temperature.
- Material was dispatched to twenty-nine laboratories of which twenty-seven laboratories (93%) returned results to the CRL.
- 31% of participating laboratories obtained 100% for all lenticules distributed.
- 41%, 48% and 72% of laboratories returned 100% correct results for norovirus GI, GII and hepatitis A respectively.
- Two laboratories did not test the lenticules for HAV**.**
- Two laboratories did not test the lenticules for NoV GI or GII.
- The false positive reporting rates for GI, GII and HAV were 3%, 4% and 1% respectively.
- The false negative reporting rates for GI, GII and HAV were 29%, 24% and 15% respectively.
- Seventeen laboratories returned quantitative real-time PCR data compared with fifteen in 2006/07.

5 References

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Appendix I - Participants' GI and GII Ct values for each lenticule

Lab ID	Lenticule 1				Lenticule 2				Lenticule 3				Lenticule 4				Lenticule 5				Lenticule 6			
	NV GI		NV GII		NV GI		NV GII		NV GI		NV GII		NV GI		NV GII		NV GI		NV GII		NV GI		NV GII	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
2	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-
3	-	-	34.1	34.9	40.9	39.3	-	-	40.2	39.6	35	32.1	-	-	33.9	35.1	-	43.2	-	-	-	-	-	-
9	-	-	27.1	24.2	37.1	34.7	31.9	39.1	38.2	37.1	-	-	-	-	41.4	39.1	44.8	37.9	-	-	36.3	34.2	-	-
10	-	-	35.7	36	37.8	37.6	40.3	-	37.9	37.2	37.7	37.2	-	-	37	36.6	38	38.5	-	-	-	-	-	-
11	-	-	41.9	42	41.4	39.8	-	-	40.8	38.6	42.2	40	-	-	44.9	41.5	40.4	39	-	-	-	-	-	-
13	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
14	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
15	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-
17	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
19	-	-	34.7	34.8	34	33.7	-	-	33.3	32.8	35	34.7	-	-	33	34.8	34	33.2	-	-	-	-	-	-
21	-	-	37.4	37	37.6	37.6	-	-	37.3	36.9	37.7	37.6	-	-	38.1	37	38.1	38	-	-	-	-	-	-
24	-	-	33.1	32.2	35.6	37	-	-	36.7	34	33.7	33.3	-	-	32.4	32.5	37	36	-	-	-	-	-	-
25	-	-	32.6	31.8	35.1	36.6	-	-	35.3	35.6	33.4	33	-	-	31.8	32.6	35	35.8	-	-	-	-	-	-
27	-	-	43.9	-	50.2	53.3	-	-	-	56.5	43.9	43.6	-	-	-	-	-	57.6	-	-	-	-	-	-
32	NE	NE	NE	NE	-	-	-	-	-	-	34.1	34.7	-	-	34.6	33.6	-	-	-	-	-	-	-	-
33	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-
35	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
37	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-
39	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39 ns	+	+			+	+			+	+			-	-			-	-			-	-		
41	-	-	32.5	33.3	33.1	-	-	-	33	-	32.1	33.6	-	-	32.4	34.7	33.1	-	-	-	-	-	-	-
43	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
47	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
48	-	-	33.6	33.8	37.7	40.5	-	-	34.7	40.4	34.3	34.4	-	-	33.9	34.1	35.7	39.2	-	-	-	-	-	-
55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
89	-	-	36.4	38.4	39.8	40.1	-	-	42.3	43.6	41	40.3	-	-	37.6	37.3	39.5	40.2	-	44.9	-	-	-	-
94	-	-	33.1	33.7	-	-	-	-	-	-	33.5	33.6	-	-	33.1	32.9	-	-	-	-	-	-	-	-
113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
133	-	-	-	40.3	40.1	38.3	-	-	39.4	37.9	41.5	39.5	-	-	41.9	39.9	40	38.4	-	-	-	-	-	-
146	-	-	+	+	+	+	-	-	-	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-

* - average Ct value recorded from 3 Ct values per replicate. ** - average Ct value recorded .from 2 Ct values per replicate.
Yellow denotes false positives, Red denotes false negatives.

Appendix II - Participants' HAV Ct values for each lenticule

Lab ID	Lenticule 1 HAV		Lenticule 2 HAV		Lenticule 3 HAV		Lenticule 4 HAV		Lenticule 5 HAV		Lenticule 6 HAV	
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2
2	-	-	+	+	+	+	+	+	-	-	-	-
3	-	-	35	34.5	33.62	33.57	33.99	33.8	-	-	-	-
9	-	-	+	+	+	+	+	+	-	-	-	-
10	-	-	34.94	35.28	36.37	35.44	34.59	34.55	-	-	-	-
11	-	-	45.36	41.7	44.96	39.8	44.34	39.82	-	-	-	-
13	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
14	-	-	+	+	+	+	+	+	-	-	-	-
15	-	-	-	+	+	+	+	+	-	-	-	-
17	-	-	+	+	+	+	+	+	-	-	-	-
19	-	-	31.97	31.74	31.72	31.49	32.04	31.83	-	-	-	-
21	-	-	35	35	34.2	34.1	34.4	34.2	-	-	-	-
24	-	-	+	+	+	+	+	+	-	-	-	-
25	-	-	28.8	29.36	29.64	29.26	28.97	29.16	-	-	-	-
27	-	-	-	-	+	+	-	-	-	-	-	-
32	NE	NE	31.4	30.9	31.7	31.5	30.8	31	-	-	-	-
33	-	-	+	+	+	+	+	+	-	-	-	-
35	-	-	+	+	+	+	+	+	-	-	-	-
37	-	-	+	+	+	+	+	+	-	-	-	-
39	-	-	+	+	+	+	+	+	-	-	-	-
41	-	-	30.45	30.67	30.07	29.89	29.82	30.09	-	-	-	-
43	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
47	-	-	33.2	34	33.5	33.4	33.2	33.4	-	-	-	-
48	-	-	43.42	38.5	39.55	38.82	38.78	39.9	-	-	-	-
55	-	-	39.55	41.45	41.85	39.35	40.38	-	-	-	-	41.93
89	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
94	-	-	31.45	32.07	31.61	31.82	30.82	30.78	-	-	-	-
113	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
133	-	-	-	42.28	43.44	40.64	42.97	38.81	-	-	-	-
146	-	-	+	+	+	+	+	+	-	-	-	-

Yellow denotes false positives, Red denotes false negatives.

Appendix III - Primers and probes used in the Taqman assay

	GI FWD	GI REV	GI Probe	GII FWD	GII REV	GII Probe	HAV FWD	HAV REV	HAV Probe
Name	QNIF4	NV1LCR	NVGGIP	QNIF2	COG2R	QNIFS			
Name	COG1F	COG1R	RING1-TP5' (6-Fam)	COG2F	COG2R	RING2-TP			
Sequence	CGYTGGATGCGNTTYCATGA	CTTAGACGCCATCATCATTYAC	AGATYGCGRTCYCCTGTCCA (BHQ1)	CARGARBCNATGTTYAGRTGGATGAG	TCGACGCCATCTTCATTCACA	5'(6-Fam)TGGGAGGGCGATCGCAATCT(BHQ1)			
Name	QNIF4	QNIF3	QNIFP	QNIF2	COG2R	QNIFs	HAV68	HAV240	HAV150(-)
Sequence	CGCTGGATGCGNTTCCAT	GTCCTTAGACGCCATCATCATTT	TGTGGACAGGAGATCGC	ATGTTCAGRTGGATGAGRTTCTCWGA	TCGACGCCATCTTCATTCACA	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA-MGB
Name	QNIF4	QNIF3	QNIFP	QNIF2	COG2R	QNIFs	HAV68	HAV240	HAV150(-)
Sequence	CGCTGGATGCGNTTCCAT	GTCCTTAGACGCCATCATCATTT	TGTGGACAGGAGATCGC	ATGTTCAGRTGGATGAGRTTCTCWGA	TCGACGCCATCTTCATTCACA	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA-MGB
Name				NV107a	NV 117	NV TM3			
Sequence				AGCCAATGTTCAGATGGATG	TCGACGCCATCTTCATTCAC	FAM-TGGGAGGGCGATCGCAATCTGGC-TAMRA			
Name	192	193	NVGG1-LNA	NV107a	NV 117	NV TM3	HAV68:	HAV240	HAV150(-)
Sequence	GCYATGTTCCGCTGGATGC	CGTCCTTAGACGCCATCATCA	FAM-ATTCGGGCAG GAGAT-EclipseDQ	AGCCAATGTTCAGATGGATG	TCGACGCCATCTTCATTCAC	FAM-TGGGAGGGCGATCGCAATCTGGC-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA -MGBNFQ
Name	QNIF4	NV1LCR	NVGG1p						
Sequence	CGCTGGATGCGNTTCCAT	CCTTAGACGCCATCATCATTTAC	FAM -TGGACAGGAG AYCGCRATCT-TAMRA						
Name	QNIF4	NV1LCR	NVGG1p	QNIF2	COG2R	QNIFs	HAV68	HAV240	HAV150(-)
Sequence	CGCTGGATGCGNTTCCAT	CCTTAGACGCCATCATCATTTAC	FAM-TGGACAGGA GAY CGCRATCT-TAMRA	ATGTTCAGRTGGATGAGRTTCTCWGA	TCGACGCCATCTTCATTCACA	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA-MGB
Name	JJV1F	COG1R	RING1(b)-TP	COG2F	COG2R	RING2-TP	HAV68	HAV240	HAV150
Sequence	GCCATGTTCCGITGGATG	CTTAGACGCCATCATCATTYAC	FAM-AGATCGCGGTC TC CTGTCCA-TAMRA	CARGARBCNATGTTYAGRTGGATGAG	TCGACGCCATCTTCATTCACA	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-TTAATTCCTGCAGGTTCAGG-TAMRA
Name	JJV1F	JJV1R	JJV1P	JJV2F	COG2R	RING2-TP	HAV68	HAV240	HAV150
Sequence	GCCATGTTCCGITGGATG	TCCTTAGACGCCATCATCAT	FAM-TGTGGACAGGAG ATCGCAATCTC-TAMRA	CAAGAGTCAATGTTTAGGTGGATGAG	TCGACGCCATCTTCATTCACA	FAM-TGGGAGGGCGATCGCAATCT-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-TTAATTCCTGCAGGTTCAGG-TAMRA
Name	QNIF4	NV1LCR	NVGG1p	QNIF2	COG2R	QNIFs	HAV68	HAV240	HAV150(-)
Sequence	CGCTGGATGCGNTTCCAT	CCTTAGACGCCATCATCATTTAC	FAM-TGGACAGGAGA YCGCRATCT-TAMRA	ATGTTCAGRTGGATGAGRTTCTCWGA	TCGACGCCATCTTCATTCACA	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA-MGB
Name	COG1F	COG1R	RING1a-TP, RING1b-TP (region ORF-1-ORF-2 junction)	COG2F	COG2R	RING2-TP(region ORF-1-ORF-2 junction)			
Name	JJVIF	JJVIR	JJVI	COG2F	COG2R	RING2			
Sequence	GCCATGTTCCGITGGATG	TCCTTAGACGCCATCATCAT	FAM –TGTGGACAGGA GATCGCAATCTC-TAMRA	CARGARBCNATGTTYAGRTGGATGAG	TCGACGCCATCTTCATTCACA	FAM-TGGGAGGGCGATCGCAATCT-TAMRA			
Name	QNIF4	QNIF3	QNIFP	QNIF2	COG2R	QNIFs	HAV68	HAV240	HAV150(-)
Sequence	CGCTGGATGCGNTTCCAT	GTCCTTAGACGCCATCATCATTT	FAM-TGTGGACAGGAG ATCGC-TAMRA	ATGTTCAGRTGGATGAGRTTCTCWGA	TCGACGCCATCTTCATTCACA	FAM-AGCACGTGGGAGGGCGATCG-TAMRA	TCACCGCCGTTTGCCTAG	GGAGAGCCCTGGAAGAAAG	FAM-CCTGAACCTGCAGGAATTAA-MGB

Appendix IV - Primers and probes used in the Nested PCR assay

	First Round		Second round		First Round		Second round		First Round		Second round	
	GI FWD	GI REV	GI FWD	GI REV	GII FWD	GII REV	GII FWD	GII REV	HAV FWD	HAV REV	HAV FWD	HAV REV
Name	G6	Y5	SR 48 SR 50 SR 52	P110	G7	Y5	NI	P110				
Sequence	GAIGGICTICCATCWGGITTYCC	ACIATYTCRTCATCICCRTARAA	GTGAACAGCATAAATCACTGG GTGAACAGTATAAACCACTGG GTGAACAGTATAAACCATTGG	AC(A/T/G)AT(C/T)TCATCATCACCATA	GARGGICTICCITCKGGIGTICC	ACIATYTCRTCATCICCRTARAA	GAATTCCATCGCCCACTGGCT	AC(A/T/G)AT(C/T)TCATCATCACCATA				
Name	GI	SM31	Ando	E3	GII	SM31	NI	E3	HAV1	HAV2	neHAV1	neHAV2
Name	GI	SM31	Ando	E3	GII	SM31	NI	E3				
Name	GI	SM31	Ando	E3	GII	SM31	NI	E3	HAV1	HAV2	neHAV1	neHAV2
Sequence	TCNGAAATGGATGTTGG	CGATTTCATCATCACCATA	GTGAACAGYATAAAYCANTGG	ATCTCATCATCACCATA	AGCCNTNGAAATNATGGT	CGATTTCATCATCACCATA	GAATTCCATCGCCCACTGGCT	ATCTCATCATCACCATA	TTGGAACGTCACCTTGCAGTG	CTGAGTACCTCAGAGGCAAAC	ATCTCTTTGATCTTCCACAAG	GAACAGTCCAGCTGTCAATGG

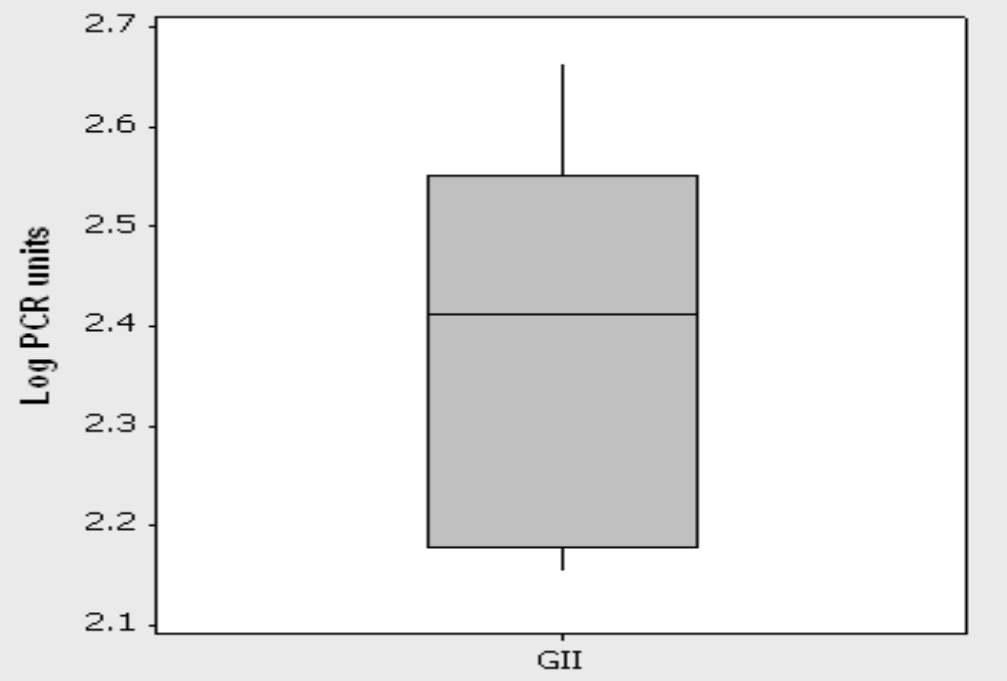
Appendix V - Primers and probes used in the One step PCR assay

	GI FWD	GI REV	GII FWD	GII REV	HAV FWD	HAV REV	Norovirus fwd	Norovirus Rev
Name	SR48F SR50F SR52F	SR33R	SR46F	SR33R	HAVF	HAVR		
Name	COG1F	COG1R	COG2F	COG2R	SH-Prot-A	SH-Prot-1		
Sequence	CGYTGGATGCGNTTYCATGA	CTTAGACGCCATCATCATTYAC	CARGARBCNATGTTYAGRTGGATGAG	TCGACGCCATCTTCATTCACA	ATGGATGCTGGRGTTCTTAC	ARTTGGCAGCAATTTCTTCAAG		
Name	COG1-F	G1-SKR	COG2-F	G2-SKR				
Sequence	CGYTGGATGCGNTTYCATGA	CCAACCCARCCATTRTACA	CARGARBCNATGTTYAGRTGGATGAG	CCRCNNGCATRHCCRTTRTACAT				
Name					HAV-F	HAV-R		
Sequence					CAGCACATCAGAAAGGTGAG	CTCCAGAATCATCTCCAAC		
Name	G1SKF	G1SKR	G2SKF	G2SKR			JV12Y	JV13I

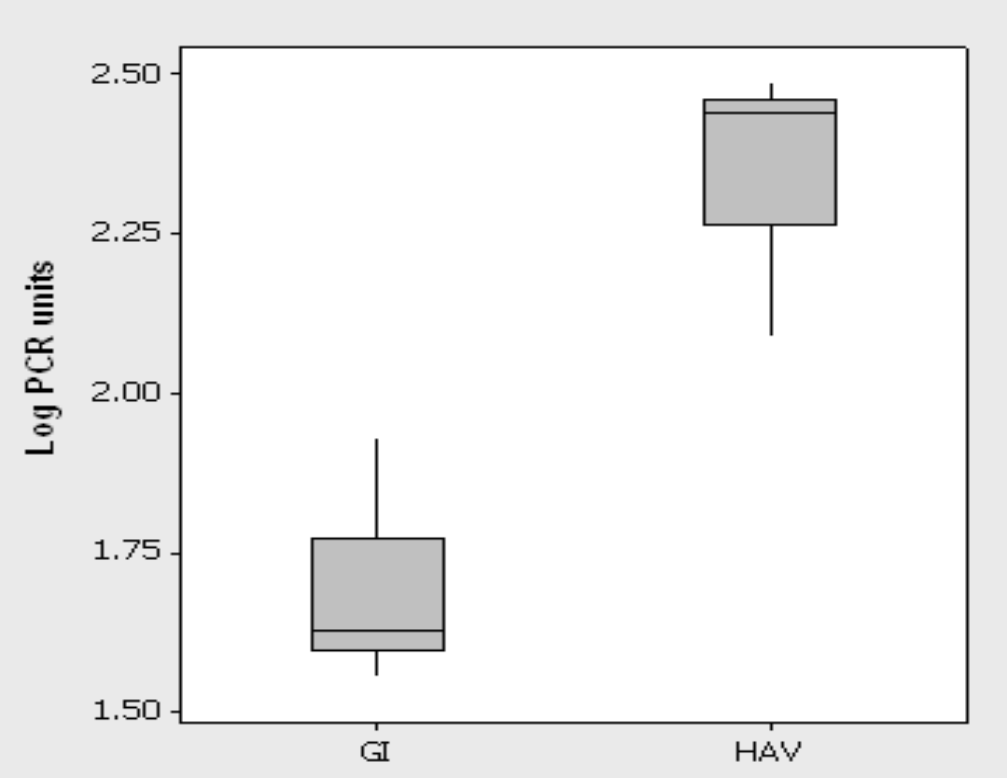
Appendix VI - Reference results

CRL Reference results displayed as boxplots.

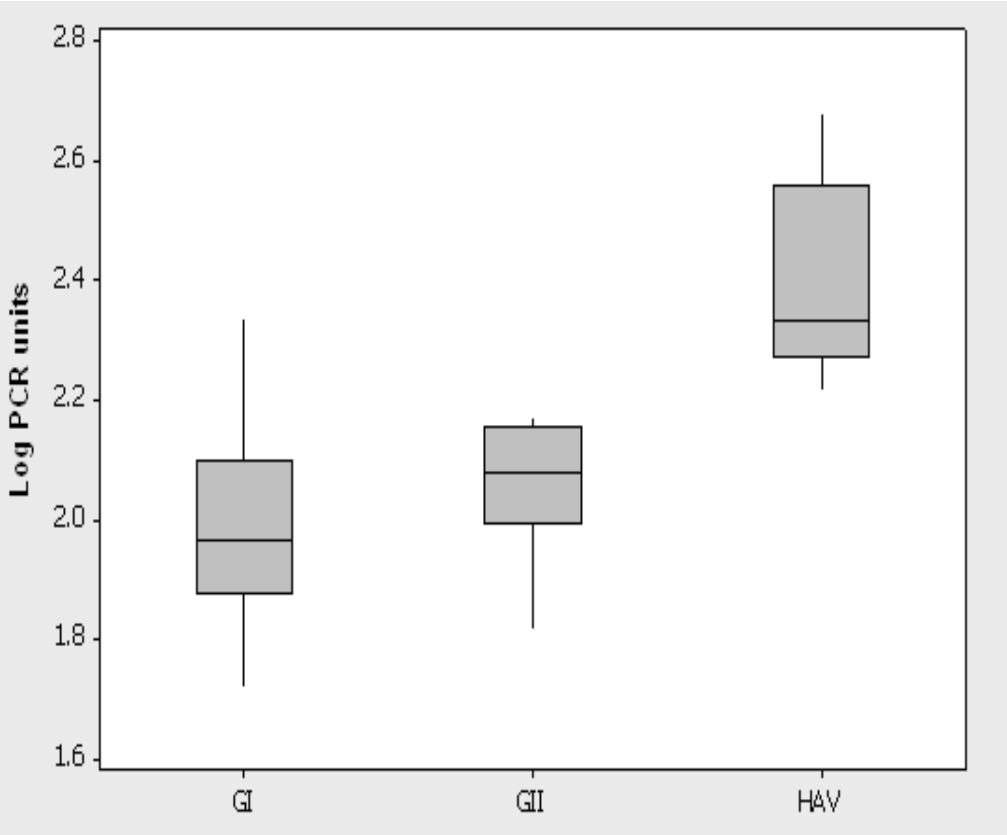
1. Lenticule RT 25 – L1



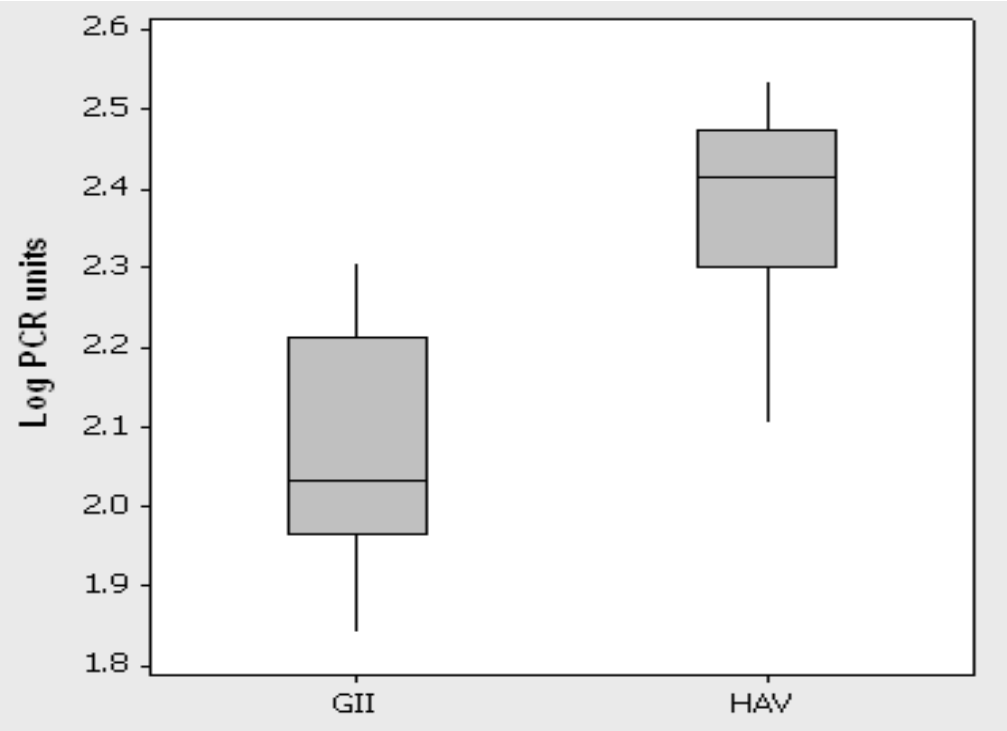
2. Lenticule RT 25 – L2



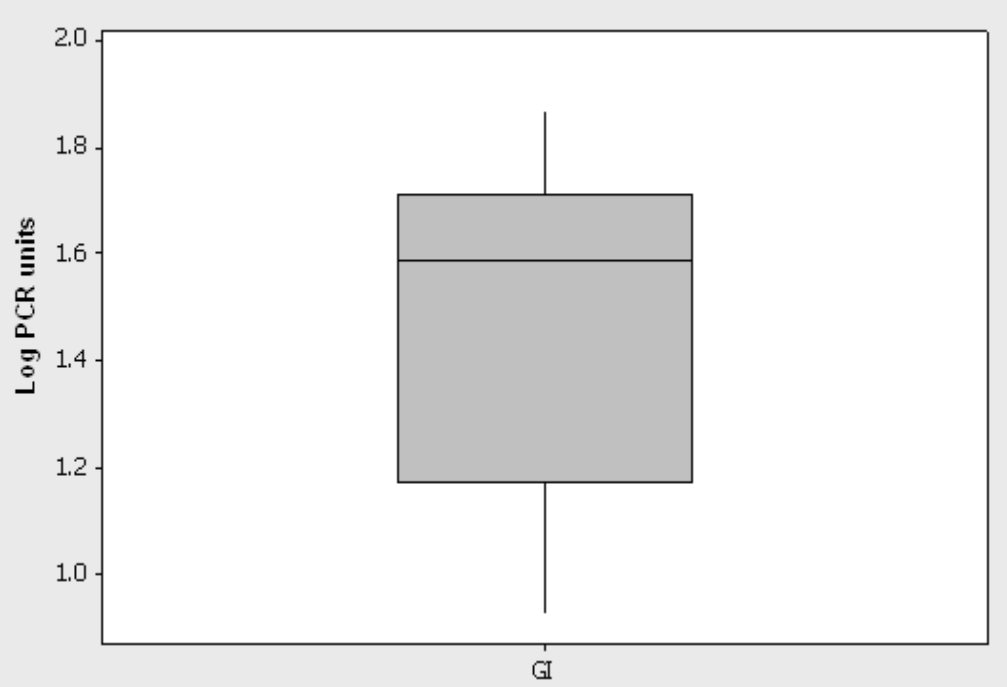
3. Lenticule RT 25 – L3



4. Lenticule RT 25 – L4



5. Lenticule RT 25 – L5



European Community Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

Report on the *Vibrio parahaemolyticus* ring trial, 2008

CRL ring trial reference: RT 22 (*V. para* 2008)

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Contents

Tables

1. Introduction

Regulation (EC) No 882/2004 designates the Centre for Environment, Fisheries and Aquaculture Science at Weymouth U.K. as the Community Reference Laboratory (CRL) for monitoring the viral and bacteriological contamination of bivalve molluscs. Under Article 32 the CRL laboratory is responsible for organising comparative testing by National Reference Laboratories.

At the 6th annual workshop of NRLs Galway 2007, several resolutions were agreed relating to proficiency testing including:

> **Resolution 32.** NRLs requested that the CRL organise a further ring trial distribution for *V. parahaemolyticus* with the intent of providing samples that enabled enumeration and determination of potential pathogenicity principles.

2. Proficiency testing samples

2.1 Sample preparation

Six samples from the Cefas *Vibrio* spp. reference strain bank were streaked onto non-selective marine agar (MA). Plates were incubated for 18-24hrs at 30±2˚C. Each strain was designated a sample code, RT 22 A to F. Following visual purity checks 2-5 colony forming units (cfu) were transferred onto sterile (ENT) swabs and stabbed in semi-solid MA. Swabs were incubated for 18-24hrs at 30±2˚C. After incubation the inoculated swabs were stored at 3±2˚C until distribution. Each participating laboratory received 6 swabs, one replicate of each strain.

Note. RT 22 consisted of samples for presence/absence testing only.

2.2 Distribution of samples

The *V. parahaemolyticus* ring trial February 2008 was designated RT 22. Samples were packaged according to IATA regulations, UN3373 as biological samples, division 6.2 under the packing instruction code 650. All participating laboratories received a single box comprising of 6 separate sealed tubes (Samples RT 22 A to F). Samples were distributed by CitySprint Ltd at ambient temperature on week commencing the 25th February 2008. On receipt, participants were requested to store the samples at 3±2˚C prior to analysis during week commencing the 10th March 2008.

2.3 Quality control at dispatch

Samples were tested prior to distribution to verify conformation to the reference strain bank designation. Analyses were undertaken using CRL standard procedures for detection of *V. parahaemolyticus* (Cefas SOP 1333), with additional biochemical tests to confirm the identification of *V. hollisae* and *V. alginolyticus*. Standard operating procedures are available from the information centre of the CRL website (www.crlcefas.org). In addition, *V. parahaemolyticus* was confirmed using *toxR* (Kim *et al* 1999) and *tlh* (McCarthy *et al* 2000). The putative pathogenicity markers were confirmed by nucleic acid hybridisation (*tdh* (McCarthy *et al* 2000) and *trh* (Nordstrom *et al.* 2006)).

Table 1. Sample analysis prior to sample distribution

Sample ID	Reference bank designation	Presence of *tdh/trh*	Reference bank accession code
RT 22 A	*V. parahaemolyticus*	no	NCTC 10885
RT 22 B	*V. alginolyticus*	no	V05/007
RT 22 C	*V. parahaemolyticus*	*tdh* only	V05/067
RT 22 D	*V. parahaemolyticus*	*tdh* and *trh*	V05/014
RT 22 E	*V. hollisae*	no	V05/006
RT 22 F	*V. parahaemolyticus*	*trh* only	E154482

2.4 Confidentiality of results

Each laboratory participant was identified by a code to preserve anonymity.

2.5 Participation

Invitations inviting expressions of interest in the *V. parahaemolyticus* ring trial were sent to all designated MS NRLs, candidate countries laboratories, Norway, Iceland and to government laboratories in the U.S and Canada. Material was dispatched to twenty-two laboratories. Table 2 summarises the participation in RT 22.

Table 2. Participation in the *V. parahaemolyticus* ring trial distribution (RT 22)

Member State	Participation in RT 22	Results returned to CRL
Austria	Yes	Yes
Belgium & Luxembourg	Yes	Yes
Bulgaria	Yes	Yes
Czech Republic	No	-
Denmark	No	-
Estonia	Yes	Yes
Finland	No	-
France	No	-
Germany	Yes	Yes
Greece	Yes	Yes
Hungary	Yes	Yes
Ireland	No	-
Italy 1	Yes	Yes
Italy 2	Yes	Yes
Latvia	Yes	Yes
Lithuania	No	-
Netherlands	Yes	Yes
Poland	Yes	Yes
Portugal	Yes	Yes
Romania	No	-
Slovakia	Yes	Yes
Slovenia	Yes	Yes
Spain	No	-
Sweden	No	-
United Kingdom	Yes	Yes

Table 2 cont. Participation in the *V. parahaemolyticus* ring trial distribution (RT 22)

Accession/ Candidate country	Participation in RT 22	Results returned to CRL
Croatia	Yes	Yes
Turkey	Yes	Yes
ETFA		
Norway	Yes	Yes
Iceland	Yes	Yes
Third country		
Canada	Yes	Yes
USA	Yes	Yes

2.6 Reference results

Reference analyses were performed by the CRL. In brief, 5 replicate samples selected at random were analysed for the species type and the presence of *tdh* and *trh* genes using CRL standard methods. These analyses were carried out concurrently with participants' analyses and the results are shown in Table 3.

Table 3. Reference results for samples RT 22 A to RT 22 F

Sample ID	API identification	*tlh*	*toxR*	*tdh*	*trh*
RT 22 A	*V. parahaemolyticus*	+	+	-	-
RT 22 B	*V. alginolyticus*	-	-	-	-
RT 22 C	*V. parahaemolyticus*	+	+	+	-
RT 22 D	*V. parahaemolyticus*	+	+	+	+
RT 22 E	*V. hollisae*	-	-	-	-
RT 22 F	*V. parahaemolyticus*	+	+	-	+

3 Results

3.1 Distribution

All samples arrived at their destinations within 48 hr of dispatch. No participants reported that samples arrived in poor condition. Most laboratories (20) stored samples at 2-6˚C, 2 stored samples at room temperature prior to commencement of tests. Storage temperature appeared to have no effect on the results.

3.2 Analysis of results

Twenty-two laboratories returned results to the CRL. Eighteen (82%) used methods that enabled detection of the putative pathogenicity marker *tdh* and *trh* genes.

3.2.1 Sample RT 22 A [expected result *V. parahaemolyticus* present; *tdh* and *trh* negative]
All laboratories correctly identified *V. parahaemolyticus* in sample RT 22 A. Eighteen laboratories applied tests for *tdh* and/or *trh*. One laboratory (Lab 6) reported the presence of *tdh,* all other laboratories returned the anticipated results.

3.2.2. Sample RT 22 B [expected result *V. parahaemolyticus* not detected*, (*sample *V. alginolyticus)*; *tdh* and *trh* negative].
All laboratories correctly identified sample RT 22 B as *V. parahaemolyticus* not detected. Eleven laboratories correctly identified the presence of *V. alginolyticus*. One laboratory incorrectly identified the presence of *V. vulnificus.*

3.2.3 Sample RT 22 C [expected result *V. parahaemolyticus* present*, tdh* positive, *trh* negative]
All laboratories correctly identified *V. parahaemolyticus* in sample RT 22 C. All laboratories that applied tests to detect *tdh* correctly identified its presence. All laboratories returning results for *trh* correctly identified it as absent.

3.2.4 Sample RT 22 D [expected result *V. parahaemolyticus* present*, tdh* positive, *trh* positive]
Sixteen laboratories (73%) correctly identified sample RT 22 D as *V. parahaemolyticus.* All laboratories that undertook pathogenicity principle testing (n=13) correctly assigned sample RT 22 D as *tdh* and *trh* positive. Six laboratories (Lab 10, 14, 19, 21, 22 and 44) reported the absence of *V. parahaemolyticus* with 3 identifying *V. vulnificus* presence in sample RT 22 D.

3.2.5 Sample RT 22 E [expected result *V. parahaemolyticus* not detected *(*sample *V.hollisae), tdh* negative, *trh* negative]
Twenty laboratories that returned results reported the absence of *V. parahaemolyticus.* However, one laboratory (Lab 6) identified sample RT 22 E as *V. vulnificus.* One laboratory (Lab 10) reported the presence *V. parahaemolyticus* in sample RT 22 E. Three laboratories (Lab 10, 17 and 39) incorrectly reported the *tdh* and/or *trh* as positive.

3.2.6 Sample RT 22 F [expected result *V. parahaemolyticus* present*, tdh* negative, *trh* positive]
All laboratories that returned results correctly identified *V. parahaemolyticus* in sample RT 22 F. Lab 6 incorrectly reported the presence of *tdh.* Four laboratories (Lab 2, 22, 42 and 44) did not detect the presence of *trh.* All other laboratories that undertook pathogenicity principle testing correctly assigned sample RT 22 F as *trh* positive and *tdh* negative.

Participants' results are shown in Tables 4 to 9, false positive and negative results are identified in yellow and red respectively. Results are provided for information only. No performance assessments were carried out.

4 Summary

In general all laboratories performed well in this trial. A false negative rate of 6.8% was observed for identification of *V. parahaemolyticus*. All false negative species identification was associated with RT 22 D. RT 22 D was a clinical strain originating from a patient with *V. parahaemolyticus* associated gastroenteritis in South East Asia. It was interesting to note that three laboratories identified this sample as *V. vulnificus* illustrating the commonly experienced problems with misidentification of some *Vibrio* spp. strains. The false positive rate was less than 1%. Eighteen laboratories used methods that enabled detection of pathogenicity markers. The majority of laboratories applying these tests correctly assigned the presence or absence of both *tdh* and *trh*. Three laboratories (Lab 6, 17 and 39) reported false *tdh* positives, and two laboratories (Lab 10 and 17) reported *trh* in an intended *trh* negative sample. Four laboratories (Lab 2, 22, 42 and 44) failed to detect *trh* in one sample.

Table 4. Participants' results for sample RT 22 A
[expected result *V. parahaemolyticus* present; *tdh* and *trh* negative]

Lab ID	Sample result	*tdh*	*trh*
2	*V. parahaemolyticus*	-	-
6	*V. parahaemolyticus*	+	-
7	*V. parahaemolyticus*	-	-
9	*V. parahaemolyticus*	-	-
10	*V. parahaemolyticus*	-	-
13	*V. parahaemolyticus*	NE	NE
14	*V. parahaemolyticus*	NE	NE
15	*V. parahaemolyticus*	-	-
17	*V. parahaemolyticus*	-	-
19	*V. parahaemolyticus*	-	-
21	*V. parahaemolyticus*	NE	NE
22	*V. parahaemolyticus*	-	-
32	*V. parahaemolyticus*	-	-
33	*V. parahaemolyticus*	-	-
35	*V. parahaemolyticus*	-	-
39	*V. parahaemolyticus*	-	-
42	*V. parahaemolyticus*	-	-
44	*V. parahaemolyticus*	-	-
55	*V. parahaemolyticus*	-	-
68	*V. parahaemolyticus*	-	-
83	*V. parahaemolyticus*	-	NE
90	*V. parahaemolyticus*	NE	NE

NE – Not examined
Yellow denotes false positive

Table 5. Participants' results for sample RT 22 B
[expected result *V. parahaemolyticus* not detected, (sample *V. alginolyticus*); *tdh* and *trh* negative]

Lab ID	Sample result	*tdh*	*trh*
2	*V. alginolyticus*	-	-
6	*V. alginolyticus*	-	-
7	*V. parahaemolyticus* not detected	-	-
9	*V. alginolyticus*	-	-
10	*V. alginolyticus*	NE	NE
13	*V. alginolyticus*	NE	NE
14	*V. parahaemolyticus* not detected	NE	NE
15	*V. parahaemolyticus* not detected	-	-
17	*V. parahaemolyticus* not detected	-	-
19	*V. alginolyticus*	NE	NE
21	*Vibrio* spp. not detected	NE	NE
22	*V. parahaemolyticus* not detected	-	-
32	*V. alginolyticus*	-	-
33	*V. alginolyticus*	-	-
35	*V. alginolyticus*	NE	NE
39	*V. alginolyticus*	-	-
42	*V. vulnificus*	-	-
44	*V. parahaemolyticus* not detected	-	-
55	*V. parahaemolyticus* not detected	-	-
68	absence of growth	NE	NE
83	*V. parahaemolyticus* not detected	NE	NE
90	*V. alginolyticus*	NE	NE

Table 6. Participants' results for sample RT 22 C
[expected result *V. parahaemolyticus* present, *tdh* positive, *trh* negative]

Lab ID	Sample result	*tdh*	*trh*
2	*V. parahaemolyticus*	+	-
6	*V. parahaemolyticus*	+	-
7	*V. parahaemolyticus*	+	-
9	*V. parahaemolyticus*	+	-
10	*V. parahaemolyticus*	+	-
13	*V. parahaemolyticus*	NE	NE
14	*V. parahaemolyticus*	NE	NE
15	*V. parahaemolyticus*	+	-
17	*V. parahaemolyticus*	+	-
19	*V. parahaemolyticus*	+	-
21	*V. parahaemolyticus*	NE	NE
22	*V. parahaemolyticus*	NE	NE
32	*V. parahaemolyticus*	+	-
33	*V. parahaemolyticus*	+	-
35	*V. parahaemolyticus*	+	-
39	*V. parahaemolyticus*	+	-
42	*V. parahaemolyticus*	NE	NE
44	*V. parahaemolyticus*	NE	NE
55	*V. parahaemolyticus*	+	-
68	*V. parahaemolyticus*	+	-
83	*V. parahaemolyticus*	+	NE
90	*V. parahaemolyticus*	NE	NE

NE – Not examined
Red denotes false negative

Table 7. Participants' results for sample RT 22 D
[expected result *V. parahaemolyticus* present, *tdh* positive, *trh* positive]

Lab ID	Sample result	*tdh*	*trh*
2	*V. parahaemolyticus*	+	+
6	*V. parahaemolyticus*	+	+
7	*V. parahaemolyticus*	+	+
9	*V. parahaemolyticus*	+	+
10	*V. vulnificus*	NE	NE
13	*V. parahaemolyticus*	NE	NE
14	*V. parahaemolyticus* not detected	NE	NE
15	*V. parahaemolyticus*	+	+
17	*V. parahaemolyticus*	+	+
19	*V. vulnificus*	NE	NE
21	*V. vulnificus*	NE	NE
22	*V. parahaemolyticus* not detected	NE	NE
32	*V. parahaemolyticus*	+	+
33	*V. parahaemolyticus*	+	+
35	*V. parahaemolyticus*	+	+
39	*V. parahaemolyticus*	+	+
42	*V. parahaemolyticus*	NE	NE
44	*V. parahaemolyticus* not detected	NE	NE
55	*V. parahaemolyticus*	+	+
68	*V. parahaemolyticus*	+	+
83	*V. parahaemolyticus*	+	NE
90	*V. parahaemolyticus*	NE	NE

Table 8. Participants' results for sample RT 22 E
[expected result *V. parahaemolyticus* not detected (sample *V.hollisae*) *tdh* negative, *trh* negative]

Lab ID	Sample results	*tdh*	*trh*
2	No growth	NE	NE
6	*V. vulnificus*	-	-
7	*V. parahaemolyticus* not detected	-	-
9	*V. parahaemolyticus* not detected	-	-
10	*V. parahaemolyticus*	-	+
13	*Pasturella spp.*	NE	NE
14	*V. parahaemolyticus* not detected	NE	NE
15	*V. parahaemolyticus* not detected	-	-
17	*V. parahaemolyticus* not detected	+	+
19	*V. parahaemolyticus* not detected	NE	NE
21	*V. hollisae*	NE	NE
22	*V. parahaemolyticus* not detected	-	-
32	*Grimontia hollisae*	-	-
33	*V. parahaemolyticus* not detected	NE	NE
35	*Aeromonas* spp.	NE	NE
39	*V. hollisae*	+	-
42	*V. parahaemolyticus* not detected	-	-
44	*V. parahaemolyticus* not detected	-	-
55	*V. parahaemolyticus* not detected	-	-
68	No growth	NE	NE
83	*V. parahaemolyticus* not detected	NE	NE
90	*V. hollisae*	NE	NE

NE – Not examined
Yellow denotes false positive
Red denotes false negative

Table 9. Participants' results for sample RT 22 F
[expected result *V. parahaemolyticus* present, *tdh* negative, *trh* positive]

Lab ID	Sample results	*tdh*	*trh*
2	*V. parahaemolyticus*	-	-
6	*V. parahaemolyticus*	+	+
7	*V. parahaemolyticus*	-	+
9	*V. parahaemolyticus*	-	+
10	*V. parahaemolyticus*	-	+
13	*V. parahaemolyticus*	NE	NE
14	*V. parahaemolyticus*	NE	NE
15	*V. parahaemolyticus*	-	+
17	*V. parahaemolyticus*	-	+
19	*V. parahaemolyticus*	-	+
21	*V. parahaemolyticus*	NE	NE
22	*V. parahaemolyticus*	-	-
32	*V. parahaemolyticus*	-	+
33	*V. parahaemolyticus*	-	+
35	*V. parahaemolyticus*	-	+
39	*V. parahaemolyticus*	-	+
42	*V. parahaemolyticus*	-	-
44	*V. parahaemolyticus*	-	-
55	*V. parahaemolyticus*	-	+
68	*V. parahaemolyticus*	-	+
83	*V. parahaemolyticus*	-	NE
90	*V. parahaemolyticus*	NE	NE

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